BIOCHEMICAL AND PHYSIOLOGICAL BASES FOR INHIBITION OF FERMENTATION IN *Zymomonas mobilis* CP4

By

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Dedicated to my beloved wife, Tahya,
for her continuous support, trust, and encouragement;
to my mother for her moral support; and
to the memory of my father.

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Abstract of Dissertation Presented to the Graduate School
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The accumulation of ethanol during fermentation is accompanied by a progressive decline in the rate of sugar conversion into ethanol and CO_2. Two factors were found to be responsible for this decline in fermentation rate in *Z. mobilis*. First, ethanol addition to the cells inhibited glycolysis by an indirect mechanism involving the plasma membrane. Ethanol decreased the effectiveness of the plasma membrane as a semipermeable barrier, allowing the leakage of essential cofactors and coenzymes. The leakage of cofactors and coenzymes reduced their availability for the glycolytic and alcohologenic enzymes. Thus leakage caused a slowdown of fermentation in *Z. mobilis*.

The second factor identified as being responsible for the decline in fermentation rate was the physiological changes in the cells during batch culture. The peak of fermentative activity (58 umoles CO_2 evolved/mg cell protein per h) occurred after the accumulation of 1.1% (w/v) ethanol (18 h) and declined to one-half this rate after 36 h (6.2%

(w/v) accumulated ethanol), although the cell number (colony forming unit) continued to increase beyond the 18 h point. An examination of the many requirements for fermentation (nucleotides, magnesium, enzyme levels, intracellular pH, and ΔpH) offered three possible explanations for the early decline in activity: (1) a decrease in the level of intracellular nucleotides, (2) a decrease in internal pH from 6.3 to 5.3, and (3) a decrease in the specific activity of two glycolytic enzymes (glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase). Phosphorus-31 NMR spectra of perchlorate extracts from cells fermenting in broth revealed very low levels of glycolytic intermediates in cells examined at the peak of fermentative activity (18 h cells) in comparison to cells examined at later stage (30 h cells), consistent with the hypothesis that glycolytic enzymes near the end of the pathway were limiting the rate of fermentation. It is likely that cell death and collapse of pH also contributed to the further decline in the *Z. mobilis* fermentative activity after 30 h.

CHAPTER I
LITERATURE REVIEW

Introduction

Zymomonas is a unique genus of bacteria with uncertain taxonomical affiliation, despite the application of the modern numerical analysis system of taxonomy and molecular biological techniques on it. Since the original naming of this organism as Termobacterium mobile by Lindner (1931), which later changed to Zymomonas mobilis, about 20 different names have been used (Swings and De Ley, 1977). The different species of this organism have been isolated from tainted cider (Barker and Hillier, 1912), pulque (Lindner, 1928), palm juice (Okafar, 1975), sugar cane juice (Gonçalves de Lima et al., 1970), ripening honey (Ruiz-Argueso and Rodrigues-Navarro, 1975), and from contaminated beer (Shimwell, 1937).

Interestingly, this organism has been brought recently to the attention of the scientific community as a result of the excellent review by Swings and De Ley (1977) and partially because of the energy crises in the early 1970s. Zymomonas is a gram-negative, rod-shaped, motile, non-spore forming, anaerobic, and microaeroduric bacterium. It contains catalase and oxidase activities. This organism has a G + C content of 48.5 ± 1%, genome size of 1.5×10^9 daltons with approximately 1500 cistrons, and is about 56% the size of the *E. coli* genome.

Growth Requirements

Swings and De Ley (1977) have tested the growth of 40 different strains of *Zymomonas* on a variety of media ranging from complex to semisynthetic. Several other semidefined media have been developed for continuous culture studies (Cromie and Doelle, 1982; Rogers et al., 1982, Skotnikie et al., 1981; Lawford et al., 1983) and four minimal media have been described for auxotroph selection (Goodman et al., 1982; Lawford et al., 1983; Galani et al, 1985; Nipkow et al., 1984). From these media and similar recipes the requirements for *Zymomonas* growth have been identified as pantothenate, thiamine, pyridoxine, biotin, magnesium, calcium, molybdenum, phosphate, sulpher and iron in addition to carbon and inorganic nitrogen. However, many strains do not have absolute requirements for all of these vitamins and metal ions.

Genetics of Zymomonas

Zymomonas is a very stable genus in spite of its worldwide distribution. Genetically, it has a close resemblance to *Gluconobacter*, *Acetobacter*, *Rhizobium*, *Rhodopseudomonas*, and *Agrobacterium* (Swings and De Ley, 1977). All strains of Zymomonas are resistant to bacitracin, gentamicin, kanamycin, lincomycin, nalidixic acid, neomycin, penicillin, polymyxin, strptomycin, ampicillin, erythromycin, methicillin, trimethoprim, and mercuric chloride. All strains are sensitive to tetracycline, rifampicin, novobiocin, fusidic acid, chloramphenical, and sulfafurazal (Swings and De Ley, 1977; Stokes et al., 1983; Skotnicki et al., 1982).

A range of mutants of Z. mobilis have been isolated that exhibited superior characteristics of industrial importance in comparison to the wild-type, such as ethanol-tolerant mutants with greater flocculation characteristics and tolerance to molasses (Goodman et al., 1982). Ultraviolet light was not found to be a suitable mutagen, but nitrosoguanidine (NTG) has been suggested as a better mutagen for Zymomonas (Skotnicki et al., 1983). The Tn-5 containing plasmid pJ4JI has been utilized for transposon mutagenesis in Zymomonas (Skotnicki et al., 1980).

Native plasmids in Zymomonas were recently isolated (Dally et al., 1982; Skotnicki et al., 1983; Rogers et al., 1982; Tonomura et al., 1982). The number and size of the plasmid varies with the strains. The plasmids range in molecular weight from 1 to 46 megadaltons and all strains tested seem to have in common a large 46-megadalton plasmid. No other phenotypic characteristics beside antibiotic resistance (Walia et al., 1984; Skotnicki et al., 1980) have been attributed to the plasmids in spite of the suggestion that sucrose utilization and levan production may be plasmid encoded (Montenecourt, 1985). Wills et al. (1981) suggested that the alcohol dehydrogenase (ZADH-II) gene is plasmid encoded. However, these hypotheses have been refuted and rejected by Skotnicki et al. (1981).

Zymomonas mobilis is capable of three- to five-fold higher rates of fermentation compared to yeast (Rogers et al., 1982). However, the range of substrate utilized by this organism is restricted to glucose, fructose, and sucrose (Swings and De Ley, 1977). Therefore, it may be possible to genetically manipulate this organism to broaden its substrate range, e.g., to allow it to utilize starch, cellulose, or lactose. These traits will make this organism more attractive for industrial applications. Recent studies have shown that plasmids of the Pl and FII incompatibility groups can be conjugally transferred into *Z. mobilis* (Cary et al., 1984; Dally et al., 1982; Skotnicki et al., 1980; Tonomura et al, 1982; Stokes et al., 1983). Except for one report by Brown et al. (1984), transformation has not yet been accomplished. Genetic manipulation of *Z. mobilis* through spheroplast fusion has been explored (Yanase et al., 1985). The authors reported a successful method for fusion of *Z. mobilis* spheroplasts, generated by cultivating two mutants lacking the ability to ferment sugars in a hypertonic medium containing penicillin G or glycine at 30°C. The fusants were able to ferment sugars, through complementation.

Growth at Different Temperatures

It has been reported that the optimum temperature for growth of various strains of Z. mobilis is between 25 and 31°C (Millis, 1956). Swings and De Ley (1977) tested the growth of 40 different strains in liquid medium at different temperatures and found that 100% of the strains grew at 30°C, 97% grew at 34-36°C, 74% grew at 38°C, and 5% grew at 40°C. Forrest (1967) reported that the two strains CP3 and CP4 were able to grow at 42°C. Growth at high temperature is a highly desirable character in fermentative organisms, to compensate for the generation of heat during large-scale fermentation. Such increases in temperature have been shown to decrease the efficiency of alcohol production and increase its toxicity; increased temperature also increased the maintenance energy coefficient (Laudrin and Goma, 1982; Lee et al., 1981; Ohta et al., 1981).

Growth at Different pH Values

Zymomonas tolerates a wide range of pH, from 3.5-7.5, with an optimum value between 5 and 7 (Swings and De Ley, 1977). Most standard growth media for this organism start with a neutral pH, which drops during the course of fermentation due to the evolution of CO_2. King and Hossain (1982) have reported that the final yield of ethanol is insensitive to extracellular pH in the range between 5 and 7.5. However, the internal pH value appears to be very important to maintain high fermentation rate (Welch and Scopes, 1985).

Ethanol Tolerance

Historically, strains of Zymomonas have been isolated from alcoholic beverages (as spoilage organisms) and fermenting saps containing 2-10% (w/v) ethanol. Swings and De Ley (1977) reported that CP4 can tolerate 10% (w/v) ethanol and is compared to yeast (Ohta et al., 1981). Effects of ethanol on cell membranes are well documented in E. coli (Eaton et al., 1982), yeast (Beaven et al., 1982), and in Z. mobilis (Ohta et al., 1981; Cary and Ingram, 1983). Ethanol induced a change in the composition of the plasma membranes of E. coli and yeast by increasing the proportion of C18:1 vaccinic acid and decreasing C16:0 content (palmitic acid), a decrease in the amount of phosphatidylethanolamine, an increase in the phosphatidylglycerol and cardiolipin, and a decrease in the lipid/protein ratio (Ingram et al., 1986). These ethanol-induced changes in fatty acid composition of E. coli and yeast were shown to be beneficial for growth and survival in the presence of ethanol. On the other hand, Zymomonas does not undergo dramatic changes in fatty acid composition in response to added ethanol or the accumulation of ethanol during batch fermentation. This organism has a membrane phospholipid in which over 70% of the fatty acids present are 18:1 vaccinic acid (Cary and Ingram, 1983). However, Bringer et al. (1985) have reported an increase in hopanoid content during accumulation of ethanol in batch culture. This organism is highly specialized for obligate ethanol production as reflected in membrane composition which helps the bacterium to tolerate high ethanol concentrations.

Inhibition of Fermentation

Some strains of *Zymomonas mobilis* have the ability to tolerate high sugar concentrations (up to 40%) and the potential for high yields of ethanol under the conditions of low yields of biomass (Swings and De Ley, 1977). *Zymomonas* is able to produce ethanol and CO_2 from glucose, fructose, and sucrose with up to 98% of the theoretical yield. The accumulation of ethanol in the surrounding environment represents a form of chemical stress, expected to be analogous to extremes in pH and temperature that are documented to cause adaptive changes in membrane compositions in *E. coli* and *S. cerevisiae* (Ingram and Buttke, 1984; Ingram et al., 1986). It has long been recognized that industrial ethanol fermentation slows down during the batch fermentation, despite the availability of the sugar substrate. For decades, numerous workers sought to define the mechanism(s) by which ethanol inhibits its own production by yeast and other microorganisms. Several hypotheses have been proposed, such as end product inhibition and inactivation of glycolytic and alcohologenic enzymes (Burrill et al., 1983; Cromie and Doelle, 1982;

Moulin et al., 1984; Millar et al., 1982), intracellular accumulation of ethanol (Laudrin and Goma, 1982; Nagodawithana and Steinkraus, 1976; Navarro and Durand, 1978), and nutrient limitations during high gravity fermentation (Casey et al., 1984). However, recent studies have shown that none of the proposed mechanisms is the actual solo cause of the progressive decline in the rate of fermentation (Dombek and Ingram, 1986; Guijarro and Lagunas, 1984; Millar et al., 1982; Moulin et al., 1984). Other studies have also reported that ethanol has many effects on the cell membranes resulting in cellular leakage of nucleotides and magnesium (Benschoter and Ingram, 1986; Ingram and Buttke, 1984).

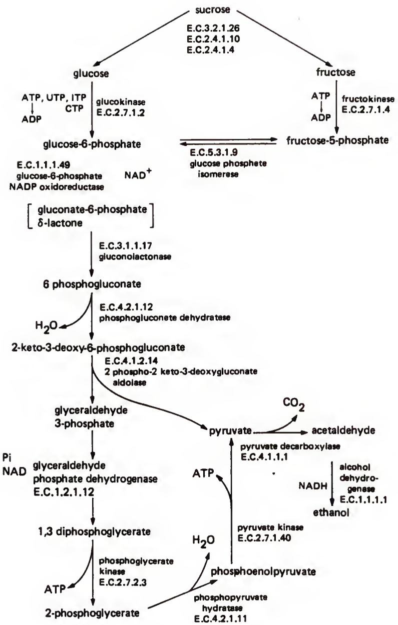
Metabolic Pathway

In the early 1950s, *Zymomonas* acquired a certain fame among biochemists by the discovery of Gibbs and DeMoss (1951, 1954) that the anaerobic catabolism of glucose in this organism follows the Entner-Doudoroff pathway rather than the Embden-Myerhoff pathway utilized by yeast. This was surprising since this pathway had been found exclusively in strictly aerobic bacteria of the genus Pseudomonas. In the case of *Z. mobilis* only one mole of ATP is produced per mole of glucose or fructose utilized. This necessitates that the organism ferment very rapidly and have very high activities of the enzymes. This discovery was later confirmed using radiorespirometric techniques (Stern et al., 1960).

Figure 1 shows that the Entner-Doudoroff pathway can be divided into three parts. Part one represents the enzymes from the moment of glucose uptake to the degradation of KDPG into pyruvate and glyceraldehyde-3-phosphate. Part two is the glycolytic enzymes which convert glyceraldehyde-3-phosphate into pyruvate. The last part of the pathway consists of alcohologenic enzymes which are responsible for the conversion of pyruvate into CO_2 and ethanol. Recently, all of the enzymes involved in glycolysis and alcohol production by Z. mobilis have been purified and partially characterized, primarily by Scopes and co-workers (1983, 1984, 1985, 1986). These enzymes have been purified using combined methods of column chromatography and differential dye-ligand binding chromatography.

Glucokinase (EC 2.7.1.2) and fructokinase (EC 2.7.1.4) were shown to be dimeric protein with apparent molecular sizes of 66,000 and 56,000 daltons, respectively (Scopes et al., 1985). Doelle (1982) has reported different sizes for these enzymes, 60,000 and 85,000 daltons, respectively. The glucokinase exhibited two pH optima at pH 7.0 and pH 8.2. It gave a hyperbolic saturation kinetics with glucose and utilized ATP, UTP, ITP, and CTP equally well (Doelle, 1982). The V_{max} for saturating substrate was estimated to be 400 $\pm$ 40 units/mg protein. The K_m values were determined to be 0.8 mM for ATP

Figure 1. Schematic representation of the modified Entner-Doudoroff pathway utilized by Zymomonas (adopted from Montenecourt, 1985).


sucrose
E.C.3.2.1.26
E.C.2.4.1.10
E.C.2.4.1.4
glucose
fructose
ATP, UTP, ITP
CTP
ADP
glucokinase
E.C.2.7.1.2
ATP
ADP
fructokinase
E.C.2.7.1.4
glucose-6-phosphate
fructose-5-phosphate
E.C.5.3.1.9
glucose phosphate isomerase
E.C.1.1.1.49
glucose-6-phosphate
NADP oxidoreductase
NAD$^+$
gluconate-6-phosphate δ-lactone
E.C.3.1.1.17
gluconolactonase
6 phosphogluconate
E.C.4.2.1.12
phosphogluconate dehydratase
H_2O
2-keto-3-deoxy-6-phosphogluconate
E.C.4.1.2.14
2 phospho-2 keto-3-deoxygluconate aldolase
glyceraldehyde 3-phosphate
pyruvate
CO_2
acetaldehyde
pyruvate decarboxylase
E.C.4.1.1.1
Pi
NAD
glyceraldehyde phosphate dehydrogenase
E.C.1.2.1.12
ATP
NADH
alcohol dehydrogenase
E.C.1.1.1.1
ethanol
1,3 diphosphoglycerate
H_2O
pyruvate kinase
E.C.2.7.1.40
phosphoglycerate kinase
E.C.2.7.2.3
phosphoenolpyruvate
ATP
2-phosphoglycerate
phosphopyruvate hydratase
E.C.4.2.1.11

at 10 mM glucose and 0.22 mM for glucose at 2 mM ATP (Scopes et al., 1985). These K_m values for ATP are significantly higher than those reported by Doelle (1982). Inhibition of activity occurred at higher level of nucleotides triphosphates and with AMP, ADP, and the end product of its reaction: glucose-6-phosphate (Scopes et al., 1982; Doelle, 1982). The fructokinase (EC 2.7.1.4) exhibited a pH optimum of 7.4 and gave hyperbolic saturation kinetics for fructose with absolute specificity for ATP. The V_{max} was 350 ± 40 units/mg protein with saturating fructose and the K_m values were calculated to be 0.45 mM for ATP at 10 mM fructose and 0.7 mM for fructose at 1 mM ATP (Scopes et al., 1985). Fructokinase was inhibited by glucose, glucose-6-phosphate, and slightly with fructose-6-phosphate (Doelle, 1982). These two enzymes were found to be exclusively responsible for sugar uptake and its subsequent phosphorylation and hexokinase with broad specificity was not found in _Z. mobilis_.

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) has been studied by several groups (Sly and Doelle, 1968; Scopes et al., 1985). This enzyme had an optimum pH of 8-8.8. The K_m for glucose-6-phosphate was 0.2-0.5 mM, 0.21 mM for NAD^+, and 0.04 mM for $NADP^+$; the V_{max} was reported to be 680 ± 50 units/mg protein. This enzyme was found to be a tetramer of similar subunits with a total molecular mass of 260,000 daltons (Montenecourt, 1985), although Scopes et al.

(1985) have reported a somewhat smaller size (208,000 daltons) for the enzyme. ATP appeared to compete with NAD^+ for binding with the enzyme. The enzyme showed a negative allosteric regulation by phosphoenolpyruvate.

6-Phosphogluconolactonase (EC 3.1.1.31) is present at high levels in Z. mobilis cells. It catalyzes the hydrolysis of 6-phosphoglucono- -lactone. It is a monomer of 26,000 daltons, has an optimum pH of 6.7, a K_m of 0.023 mM, and a V_{max} 4600 units/mg protein (Scopes, 1985).

6-Phosphogluconate dehydratase (EC 4.2.1.12) enzyme is specific to the Entner-Doudoroff pathway, and it catalyzes an essentially irreversible reaction, under physiological conditions. It contains iron and is rapidly inactivated in the absence of reducing agents (oxidizing conditions). It requires manganous and ferrous ions for stability and activation. The enzyme is a dimer and has a molecular weight of 130,000 daltons. It is inhibited by glycerophosphate, phosphate, 3-phosphoglycerate, and sulfate, whereas adenine nucleotides had no effect on the activity. The K_m was 0.04 + 0.1 mM (Scopes and Griffith-Smith, 1984).

Another enzyme that is specific to the Entner-Doudoroff pathway is 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14). It is a trimer with a molecular mass of 70,000 ± 4000 daltons. The K_m for KDPG was found to be 0.25 ± 0.04 mM. The amount of this enzyme present in the cells is at least 5 times in excess of that needed to account for the net flux through the metabolic sequence. The one-step purification procedure gave an enzyme with a specific activity of 600 units/mg (Scopes, 1984).

Most sugar-fermenting pathways in bacteria involve the five enzymes from glyceraldehyde-3-phosphate dehydrogenase to pyruvate kinase, whether the overall route is by Embden-Meyerhoff pathway or Entner-Doudoroff pathway. Pawluk et al. (1986) have reported the properties of these five enzymes from _Z. mobilis_.

Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) is a tetramer has a molecular weight of 150,000 ± 10,000 daltons. The enzyme lost activity rapidly without thiol protection, i.e., absence of β-mercaptoethanol from the assay buffer. Nevertheless, completely an inactive preparation that had been stored for several months as $(NH_4)_2SO_4$ suspension was re-activated within minutes on addition of a thiol compound.

Phosphoglycerate kinase (EC 2.7.2.3) is a monomer with a molecular size of 44,000 daltons. Its specific activity is between 700-900 units/mg, with a much less pronounced substrate-activation effect than occurs with the yeast enzyme.

Phosphoglycerate mutase (EC 5.4.2.1) is 2,3-bisphosphoglycerate-dependent, but unlike yeast enzyme it exhibited 20% activity with 3-phosphoglycerate preparation that allowed no activity with yeast enzyme in the absence of 2,3-bisphosphoglycerate. Its specific activity of 2000 units/mg protein is very high. Although it is the glycolytic enzyme of greatest activity in Z. mobilis extracts, as a protein it is by no means the most abundant. It is a dimer has a molecular size of 52,000 ± 2000 daltons. The K_m for 3-phosphoglycerate was determined to be 1.1 nM.

Enolase (EC 4.2.1.11) makes up about 2% of the protein in crude extracts from Z. mobilis, although its activity was only 180 units/mg protein. It is an octameric enzyme with a molecular size of 300,000 daltons. The K_m for 2-phosphoglycerate was 0.08 mM, a value comparable with that of the yeast enzyme.

In contrast to pyruvate kinase from other organisms, Z. mobilis pyruvate kinase (EC 2.7.1.40) was found not to require K^+ ions. The specific activity of the purified enzyme was 300 units/mg protein. The K_m for ADP (at 1 mM-phosphoenolpyruvate) was 0.17 mM, and that for phosphoenolpyruvate (at 1 mM-ADP) 0.08 mM. Unlike the tetrameric pyruvate kinase from other sources, this enzyme is a dimer with a molecular size of 115,000 ± 5000 daltons.

Pyruvate decarboxylase (EC 4.1.1.1) from Z. mobilis was partially purified and characterized by Hoppner and Doelle (1983). The molecular weight was estimated to be 219,700 ± 20,400 daltons; it exhibited two pH optima with a major peak at pH 6.0 and a minor peak at 4.3. The K_m for pyruvate was 4.4 mM at pH 6.0 and 23 mM at pH 4.3. Calcium chloride (0.2 mM) increased the activity of pyruvate decarboxylase 400-fold. Ethanol, NAD^+, ATP, ADP, AMP, and the sulphydryl binding reagents (tested) exhibited no effect on pyruvate decarboxylase activity.

Alcohol dehydrogenase (EC 1.1.1.1) from Z. mobilis was found to be activated by ferrous ions but not by zinc (Scopes, 1983) unlike alcohol dehydrogenases isolated from other bacteria (Bridgen et al., 1973), yeasts (Coleman and Weiner, 1973; Crudel and Iwatsubo, 1968), and liver (Maret et al., 1979; Shnneider-Bernlohr et al., 1981). This contrasts with early reports by Wills et al. (1981), Hoppner and Doelle (1982), and Kinoshita (1985) where each of the two Zymomonas isoenzymes (ZADH-I and ZADH-II) contained zinc. ZADH-I is a tetramer with a molecular size of 160,000 daltons while ZADH-II is also a tetramer with a molecular size of 150,000 daltons. ZADH-I reduces acetaldehyde with a pH optimum at 6.5; this reduction was faster in the presence of ethanol, an example of "product activation." ZADH-I had a V_{max} for acetaldehyde reduction 2.2 times faster than for ethanol oxidation. ZADH-II has a pH optimum of 8.5 for acetaldehyde reduction that is much slower than ethanol oxidation. The pH optimum for ethanol oxidation was 9.5. This isoenzyme is responsible for 90% of the ethanol-oxidizing ability with almost no activity towards butanol. Kinoshita et al. (1985) reported that the molecular sizes for ZADH-I and ZADH-II were 80,000 and 98,000, respectively, and each isoenzyme was a dimer. The optimal pH of ZADH-I in ethanol oxidation was 9.5 and for acetaldehyde reduction and 4.5, while those of ZADH-II were 9.5 and 6.5, respectively. ZADH-I had a V_{max} for ethanol-NAD^+ and acetaldehyde-NADH of 190 and 2,300 units/mg protein; while the K_m's were 4800 and 86 uM, respectively. ZADH-II had a V_{max} for ethanol-NAD^+ and acetaldehyde-NADH of 820 and 11,000 units/mg protein and K_m's of 2700 and 1300 uM, respectively. Wills et al. (1981) have reported the ZADH-I as a tetramer of 140,000 daltons and ZADH-II as a dimer of a molecular mass of 62,000 daltons. The pH optima for ZADH-I was 9.5 and that for ZADH-II was 10.

These enzymes are required for the fermentative production of ethanol and the rates of the reactions they catalyze in vivo define the glycolytic flux. In addition to these enzymes fermentation requires coenzymes (TPP, ATP, ADP, AMP, NAD^+, NADH, $NADP^+$, and NADPH), cofactors (magnesium, phosphorus, etc.), optimum growth temperature and pH, an internal pH value that is optimum for enzymes activity, adequate supply of sugar substrate, and a semipermeable membrane that controls the entry of the substrate and the exit of the waste end products.

In these studies we have investigated the acute effects of ethanol on the fermentation rate and the more complex changes which occur during batch fermentative accumulation of high levels of ethanol. In an attempt to define the biochemical and physiological basis for this inhibition of fermentation in Zymomonas, I have studied the effects of added ethanol on the rate of fermentation and the intracellular levels of all the Entner-Doudoroff pathway enzymes, coenzyme levels, cofactor levels, and the level of the intermediary metabolites.

CHAPTER II
MECHANISM OF ETHANOL-INHIBITION OF FERMENTATION IN *Zymomonas mobilis* STRAIN CP4

Introduction

Zymomonas mobilis is an obligately fermentative bacterium that produces ethanol and carbon dioxide as principal fermentation products (Swings and De Ley, 1977). This organism is of considerable interest for the commercial production of ethanol and has been reported to be capable of sugar conversion rates several-fold those of *Saccharomyces cerevisiae* under optimal conditions (Lawford et al., 1983; Rogers et al., 1980).

The renewed interest in ethanol as a bio-fuel, octane-enhancer, and chemical feedstock has led to numerous investigations into methods for increasing the rates and yields of fermentation by both *S. cerevisiae* (Aiba et al., 1968; Beaven et al., 1982; Ohta and Hayashida, 1983; Sedha et al., 1984; Thomas and Rose, 1979) and *Z. mobilis* (Burrill et al., 1983; Millar et al., 1982; Moulin et al., 1984; Rogers et al., 1980; Sterhaiano and Goma, 1981). One of the problems associated with large scale ethanol production is the decrease in the rate of substrate conversion observed during ethanol accumulation in the medium (Aiba et al., 1968; Laudrin and Goma, 1982; Moulin et al., 1984; Nagodawithana and Steinkraus, 1976). Despite centuries of experience in the art of fermentation (Demain and Solomon, 1981; Harden, 1923), the mechanism for the inhibition of fermentation by ethanol has not been established. Current hypotheses concerning this mechanism have proposed feedback inhibition or inactivation of enzymes which are involved in alcohol production (Hoppnor and Doelle, 1983; Laudrin and Goma, 1982; Millar et al., 1982; Nagodawithana and Steinkraus, 1976). However, these hypotheses are not fully supported by *in vitro* studies of enzyme activity and stability (Millar et al., 1982; Moulin et al., 1984).

In this study, we have developed a different approach to investigate the mechanism for ethanol-inhibition of fermentation in *Z. mobilis*. This approach involves the treatment of cells suspended in buffer or growth medium as if they were enzymes. The consumption of glucose was monitored as a measure of fermentation. Our results support the hypothesis that the principal action of ethanol which results in a decreased rate of fermentation is to increase membrane leakage, reducing the intracellular concentrations of cofactors and coenzymes essential for the activity of enzymes involved in glycolysis and alcohol production.

Materials and Methods

Organism and Growth Conditions

The organism used in these studies, *Z. mobilis* strain CP4, was generously supplied by Dr. A. Ben-Bassat of Cetus Corporation (Berkeley, CA). This organism was cultivated in the medium described by Skotnicki et al. (1981) with various levels of glucose. Cells were maintained on medium solidified with 1.5% agar. Growth was monitored by measuring O.D. at 550 nm using a B&L Spectronic 70 spectrophotometer with 10 mm round cuvettes (disposable 10 mm culture tubes). For ethanol-inhibition studies, cells were harvested in exponential phase (O.D. at 550 nm of 0.7; approximately 4×10^8 cells/ml; 175 ug cell protein/ml).

Analytical Methods

Glucose was measured using the glucose oxidase assay (Raabo and Terkildsen, 1960) supplied by the Sigma Chemical Company (St. Louis, MO). Magnesium was measured using the "60-Second Magnesium" reagent system supplied by the American Monitor Corporation (Indianapolis, IN) designed for clinical determinations. Nucleotides were estimated by measuring absorbance at 260 nm. Spectra did not contain shoulders corresponding to proteins at 280 nm and did not contain measurable amounts of protein. Cell protein was measured using the procedure of Lowry et al. (1951) as described by Layne (1957). Tritium, ^{32}P and ^{14}C were measured using a Beckman Model 8000 scintillation counter with Scinti Verse II as a cocktail (Fisher Scientific Company, Fair Lawn, NJ).

Assay of Fermentation Rate

The rate of fermentation was measured by determining glucose consumption using whole cells suspended in buffer or in growth medium. Preliminary experiments were done to determine the pH optimum (pH 6.0) in sodium phosphate, sodium citrate, and HEPES-HCl buffers. Phosphate buffer was chosen for the assay system because it is a component of the growth medium and supported high rates of fermentation. Growth conditions were optimized (30°C, unshaken tube cultures containing 0.2% glucose, harvesting in exponential growth immediately before glucose exhaustion, O.D. 550 nm of 0.65-0.70) to obtain reproducible and highly active fermenting cells. Cells were harvested and washed twice in either 50 mM sodium phosphate buffer (pH 6.0) or growth medium lacking glucose using an unrefrigerated centrifuge (8,000 x g; 5 min). Standard assay conditions were growth medium or 50 mM sodium phosphate buffer (pH 6.0), 40 ug/ml of cell protein and 0.6 mM glucose. Ethanol, nucleotides, yeast extract, and other salts were also present in various experiments. Assays were carried out at 30°C and were initiated by adding glucose. Samples were removed at 10 minute intervals over a 40 minute period and inactivated by boiling for 2 min. Screw-capped tubes with teflon liners were used when high concentrations of ethanol (10-20% w/v) were present. Residual glucose was measured using the glucose oxidase assay. Linearity of this assay system was confirmed for protein concentration and time. Typical activities obtained were 15-16 umoles of glucose consumed per hour per mg cell protein in buffer and 18-20 umoles of glucose consumed per hour per mg cell protein in growth medium.

Determination of Intracellular Ethanol Concentration

Intracellular concentrations of ethanol were determined for washed cells suspended in buffer alone, in buffer containing 1.7 M ethanol (8% w/v, 10 min exposure) and in cells which had previously been exposed to 1.7 M ethanol for 10 min, centrifuged and resuspended in buffer lacking ethanol. In these determinations, cell pellets were resuspended in 4 ml of buffer per gram wet weight. One-milliliter samples in microfuge tubes were mixed with 10 uliters of ^{14}C-sorbitol (8.3 uCi/ml, 50 uCi/millimole) dissolved in tritiated water (198 uCi/ml) and allowed to equilibrate for 10 min at room temperature. Samples (50 uliters each) were removed from these suspensions for counting. The cell suspensions were then centrifuged in a Eppendorf microcentrifuge for 2 min and samples removed from the supernatants for counting. The ^{14}C/tritium ratio of the suspension divided by that of the cell-free supernatant represents the fraction of aqueous volume surrounding the cells (intercellular volume). The intracellular volume is equal to one minus the intercellular volume. Ethanol was measured in the suspension and in the supernatant using a Tracor 560 gas chromatograph equipped with an electronic integrator as described by Goel and Pamment (1984). Intracellular ethanol was calculated using the formula: ethanol concentration in the suspension = ethanol concentration in the supernatant x fractional intercellular volume + ethanol concentration within the cell x fractional intracellular volume. All radioactive compounds were purchased from the Amersham Corporation (Arlington Heights, IL).

Fractionation of Yeast Extract

Two methods were used to fractionate yeast extract (Difco Laboratories, Detroit, MI). In the first, 5 g of yeast extract was dissolved in 500 ml of distilled water and treated three times with 100 g of Norit A activated charcoal (Matheson, Coleman & Bell, East Rutherford, NJ) for 1 h each with gentle agitation. Charcoal was removed by centrifugation (8000 x g). The second procedure involved ashing 5 g of yeast extract at 500°C for 48 hr followed by dissolving the inorganic residue in distilled water.

Effects of Ethanol on Leakage

Ethanol-induced leakage of magnesium, nucleotides, and protein was determined by resuspending cells (800 ug protein/ml) in either 50 mM sodium phosphate buffer (pH 6.0) or growth medium containing a series of ethanol concentrations but lacking glucose. Cells were incubated for 30 min at 30°C, pelleted by centrifugation for 2 min in an Eppendorf microcentrifuge, and the supernatants analyzed. Magnesium was measured as indicated under analytical methods. Nucleotide leakage was measured in two ways. In buffer, nucleotide concentration was measured on a relative basis as absorbance at 260 nm. In both buffer and in growth medium, cells were prelabelled for five generations in complex medium by growth with ^{32}P-phosphate (80 uCi added per 300 ml culture containing 0.7 mM added monobasic potassium phosphate); cells were washed and resuspended to measure leakage. After the removal of cells by centrifugation, perchloric acid (10% final concentration) was added to the supernatant and the samples centrifuged a second time to eliminate nucleic acids prior to removing 0.1 ml samples for nucleotide measurement. Protein leakage was measured using cells prelabelled for five generations in complex growth medium supplemented with tritiated leucine (10 uCi, specific activity 72 Ci/millimole, added to 300 ml broth culture). Cells were washed and resuspended in various concentrations of ethanol to determine the influence of ethanol on protein leakage into the supernatants. Leakage values for protein and for magnesium are expressed as a percentage of that released into the supernatant by vortex mixing suspensions for 30 sec with chloroform (0.2 ml per 2 ml of suspension) (Benschoter and Ingram, 1986). This treatment released 70% of the total cellular protein and 80% of the total magnesium. Nucleotide values are expressed as absorbance at 260 nm and as a percentage of the total nucleotides released by 10% perchloric acid at 0°C during a 30 min period.

Results

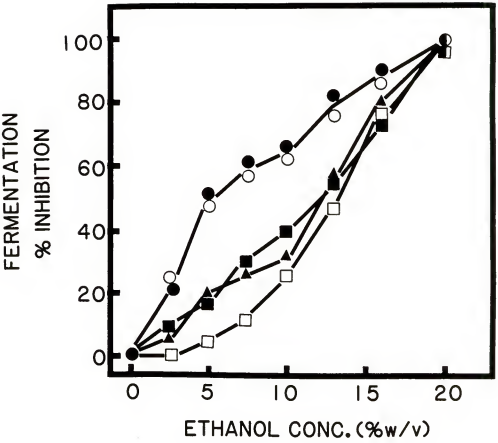
Effects of Ethanol Addition on Fermentation Rates

The addition of ethanol to Z. mobilis cells caused a dose-dependent inhibition of fermentation in both buffer and in growth medium (Fig. 2). However, fermentation was much more sensitive to inhibition by low concentrations of ethanol in buffer than in growth medium. Inhibition of 50% of fermentative activity was caused by 1.1 M ethanol (5% w/v) in buffer and 2.2 M ethanol (10% w/v) in growth medium. Fermentation was completely inhibited by the highest concentration of ethanol tested, 4.4 M (20% w/v), in both buffer and growth medium.

Ethanol Removal by Washing

I have determined the intracellular concentrations of ethanol in cells as controls for experiments involving ethanol addition and removal. The intracellular ethanol concentration was measured in washed cells suspended in buffer, in cells suspended in buffer containing 1.7 M

Figure 2. Effects of added ethanol on fermentative activity. Symbols: ● , cells assayed in 50 mM sodium phosphate buffer (pH 6.0) containing ethanol; ○ , cells exposed to various concentrations of ethanol in buffer at 30°C for 10 min, washed and assayed in buffer lacking ethanol; ▲ , cells exposed to ethanol in buffer, washed and assayed in buffer supplemented with 5 mM magnesium sulfate lacking ethanol; ■ , cells assayed in growth medium containing ethanol; □ , cells exposed to ethanol in growth medium at 30°C for 10 min, washed and assayed in growth medium lacking ethanol.


FERMENTATION
% INHIBITION
100
80
60
40
20
0
0
5
10
15
20
ETHANOL CONC.(%w/v)

ethanol (8% w/v), and in cells exposed to 1.7 M ethanol immediately after pelleting and resuspending in fresh buffer lacking ethanol. The initial ethanol concentration was less than 0.01 M. The intracellular ethanol concentration was 1.5 M (s.d. 0.2) for cells suspended in buffer containing 1.7 M ethanol and 0.02 M (s.d. 0.01) ethanol in cells exposed to ethanol and then resuspended in fresh buffer lacking ethanol. These results indicated that added ethanol was rapidly equilibrated within Z. mobilis cells and was efficiently removed by washing (Table 1).

The reversability of the inhibition of fermentation by ethanol was examined after ethanol removal by pelleting cells and resuspending them in fresh medium. The removal of ethanol from cells suspended in buffer did not cause an increase in fermentative activity (Fig. 2). The removal of ethanol from cells exposed to ethanol in growth medium resulted in a partial recovery of fermentative activity in all but the highest ethanol concentration (4.4 M). These results indicated that exposure to ethanol in buffer and to a lesser extent in growth medium resulted in irreversible damage to fermentative activity.

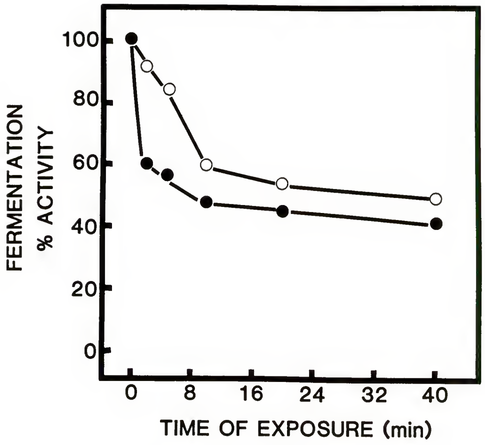
The effects of ethanol exposure time on the extent of inhibition of fermentation were examined using cells suspended in buffer and in growth medium (Fig. 3). Ethanol-damage, as measured by a reduction in fermentative activity, decreased more rapidly in buffer than in growth medium. Under both conditions, loss of fermentative activity with time was biphasic with a rapid loss during the initial

Table 1. Measurements of the internal ethanol concentration in *Z. mobilis*.

	Cell Suspension				Supernatant			
	$^{14}C/^{3}H$		[E] (% v/v)		$^{14}C/^{3}H$		[E] (% v/v)	
	Value	SD	Value	SD	Value	SD	Value	SD
unwashed 10% (v/v)	0.082	0.0017	9.5	0.085	0.104	0.0029	10.2	0.19
washed 10% (v/v)	0.085	0.005	0.696	0.029	0.107	0.003	0.853	0.097

	Interstitial volume (ml; X)	Cell volume (ml; 1-X)	$[E]_{super} \cdot X$	Internal [E] (% v/v)
unwashed cells	0.7885	0.2115	8.04	6.9
washed cells	0.7944	0.2056	0.678	0.09

Figure 3. Time-dependence of ethanol exposure on the inhibition of fermentation. Cells were suspended in ethanol solutions at 30°C. Samples were removed at various times, centrifuged, resuspended in the absence of ethanol, and assayed for fermentative activity. Symbols: ● , cells exposed to 1.1 M ethanol (5% w/v) in 50 mM sodium phosphate buffer (pH 6.0) and assayed in buffer lacking ethanol; ○ , cells exposed to 2.2 M ethanol (10% w/v) in growth medium and assayed in growth medium lacking ethanol.


FERMENTATION
% ACTIVITY
100
80
60
40
20
0
0
8
16
24
32
40
TIME OF EXPOSURE (min)

10 min of exposure followed by a slower rate of decline during the subsequent 30 min period examined.

Repair of Fermentative Activity During Incubation after Ethanol Removal

I examined the ability of *Z. mobilis* cells exposed to 1.1 M ethanol (30°C for 10 min) in buffer to regain fermentative activity after ethanol removal (Fig. 4). The fermentative activity of ethanol-damaged cells which had been resuspended in buffer continued to decline in the absence of ethanol. In contrast, the fermentative activity of ethanol-damaged cells which had been resuspended in growth media showed progressive improvement with time reaching 70% of original activity after 2 h. Cell mass was monitored spectrophotometrically during the incubation period and did not increase. Thus the improvement in fermentative activity observed in growth medium appears to result from repair of ethanol damage rather than cell growth.

The various components of growth medium were tested individually in buffer for their ability to facilitate the repair of ethanol-damage to fermentation (Table 2). Growth medium contained potassium phosphate, glucose, and yeast extract. Of these, only yeast extract was required for the partial recovery of fermentative activity. Unfortunately, yeast extract is a very complex nutrient and contains appreciable amounts of the other medium components as well.

Figure 4. Recovery of cells from ethanol-damage. Cells were exposed to 1.1 M ethanol (5% w/v) in 50 mM sodium phosphate buffer (pH 6) for 10 min at 30°C, centrifuged, and resuspended in either buffer (pH 6.0) or in growth medium. These suspensions of ethanol-damaged cells were incubated for 2 h at 30°C. Samples were removed at 30 min intervals, centrifuged, and suspended in buffer for the measurement of fermentative activity. Optical density at 550 nm was monitored as a measure of growth during incubation and did not increase in buffer or growth medium during the 2 h incubation period. Symbols: ● , cells incubated in growth medium; ○ , cells incubated in buffer.

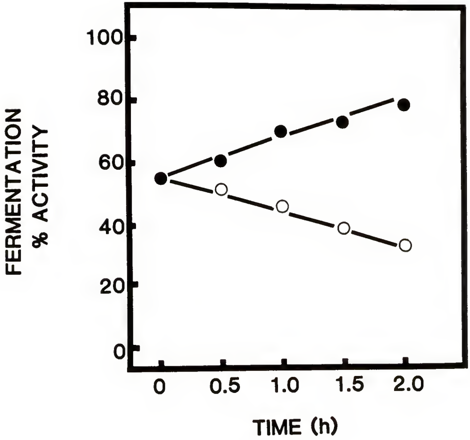

FERMENTATION
% ACTIVITY
100
80
60
40
20
0
0
0.5
1.0
1.5
2.0
TIME (h)

Table 2. Effects of growth media components on the recovery of fermentation in ethanol-damaged cells.

Treatment	Fermentative Activity	
	umole/mg protein*h	% Control
Control cells	15.0	100
Ethanol-damaged cells assayed in[a]		
buffer	5.2	35
complete medium	11.0	73
buffer + glucose (2 g/l)	7.6	51
buffer + KCl (2 g/l)	7.8	52
buffer + yeast extract (10 g/l)	10.8	72
buffer + charcoal-treated[b] yeast extract	10.5	70
buffer + ashed[b] yeast extract	11.1	74

[a] Cells were exposed to 1.1 M ethanol (5% w/v) in 50 mM sodium phosphate buffer (pH 6.0) for 10 min. Ethanol was removed by centrifugation and resuspension in buffer. Cell suspensions were then incubated for 2 h at 30°C to allow repair of ethanol-damage, centrifuged, and assayed for fermentative activity in buffer.

[b] Charcoal-treated yeast extract and ashed yeast extract were added at concentrations equivalent to that of whole yeast extract.

Yeast extract was fractionated in two ways: by treatment with charcoal to remove nucleotides, vitamins, and a variety of hydrophobic organic compounds and by ashing which destroyed all but the inorganic components. Both of these preparations were as effective as whole yeast extract (Table 2), indicating that only the inorganic components of yeast extract were required for the repair of ethanol-damage to fermentation.

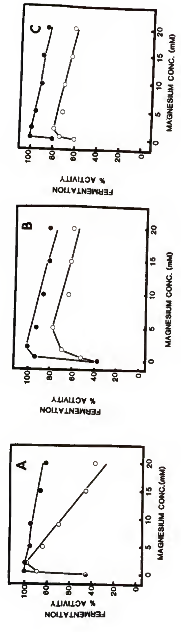
Restoration of Fermentative Activity in Ethanol-Damaged Cells by the Addition of Divalent Metals

The cellular requirements for fermentation in *Z. mobilis* are relatively simple: sugar substrate, functional enzymes (glycolytic enzymes, pyruvate decarboxylase, and alcohol dehydrogenase), coenzymes (NAD/NADH; ATP/ADP), cofactors (magnesium, and possibly calcium and/or potassium, and phosphate), and an appropriate pH and temperature. In addition, fermentation requires a semipermeable membrane which retains all essential metabolites and enzymes while permitting the entry of substrate by facilitated diffusion (Dimarco and Romano, 1985) and exit of end products. By comparing the requirements for fermentation to the results obtained with fractionated yeast extract, it is clear that only the inorganic cofactors can be supplied by the ash. Phosphate was abundant in the buffer used and the addition of potassium chloride did not support appreciable repair leaving magnesium and calcium as possible inorganic components of yeast extract which are required for the repair of ethanol-damage to fermentation.

Figure 5 shows the effects of added magnesium sulfate on fermentation rate in buffer and in growth medium. With washed cells that had been exposed to 1.1 M ethanol or 2.2 M ethanol in buffer, fermentation was completely restored by 0.5 mM magnesium sulfate and 2 mM magnesium sulfate, respectively. Concentrations of magnesium sulfate above 2 mM decreased the fermentation rate of ethanol-damaged cells in buffer. The fermentative activity of cells exposed to 2.2 M ethanol in growth medium was restored by the addition of 0.5 mM magnesium sulfate. Again, concentrations of magnesium sulfate above 2 mM caused a decrease in fermentative rate. Magnesium chloride was equally effective on a molar basis eliminating sulfate as an important component for repair.

The effects of added magnesium sulfate were also examined on fermentative activity in the continuous presence of ethanol (Fig. 5). The addition of 2 mM magnesium sulfate completely eliminated the inhibitory effect of 1.1 M ethanol in buffer and reduced the inhibitory effect of 2.2 M ethanol M in growth medium. In buffer containing 2.2 M ethanol, 5 mM magnesium sulfate was optimal for fermentation. The fermentation rate of cells in growth medium was inhibited less than 10% in growth medium containing 1.1 M ethanol and this was reversed by the addition of 0.5 mM magnesium sulfate (not shown). In the presence of ethanol,

Figure 5. Effects of added magnesium sulfate on the inhibition of fermentation by ethanol. Cells were either exposed to ethanol in 50 mM sodium phosphate buffer (pH 6.0) or in growth medium for 10 min at 30°C and assayed in the presence of ethanol or centrifuged after ethanol exposure and assayed in the absence of ethanol. Various concentrations of magnesium sulfate were added to the assay solutions. A: Buffer with 1.1 M ethanol (5% w/v); B: Buffer with 2.2 M ethanol (10% w/v); C: Growth medium with 2.2 M ethanol (10% w/v). Symbols: ○, cells in continuous presence ethanol; ●, cells exposed to ethanol and assayed after ethanol removal.


A
B
C
FERMENTATION % ACTIVITY
100
80
60
40
20
0
MAGNESIUM CONC. (mM)
0
5
10
15
20

concentrations of magnesium sulfate above 2 mM caused a progressive reduction in fermentation rate.

We have also examined the divalent metal ion specificity for the improvement of fermentative activity in the presence of ethanol and in cells which had been washed after exposure to ethanol. Comparisons were made with the addition of 0.5 mM, 1.0 mM, and 2.0 mM chloride and/or sulfate salts in buffer (1.1 M ethanol) and in growth medium (2.2 M ethanol). Magnesium was the most effective cation in restoring fermentative activity followed closely by manganese. Zinc provided a slight improvement while calcium and cobalt showed no enhancement (Table 3).

To further examine the significance of divalent metals such as magnesium as an explanation for the differences in the ethanol-sensitivity of fermentation in buffer and in growth medium, I re-examined the inhibition of fermentation by ethanol in buffer supplemented with magnesium sulfate (Fig. 2). Cells suspended in buffer containing 5 mM magnesium sulfate exhibited a decreased sensitivity to inhibition of fermentation by ethanol, equivalent to that of growth medium. Further, the addition of 10 mM ethylenediamine tetraacetic acid (EDTA) to growth medium increased the extent of inhibition of fermentation by 2.2 M ethanol (10% w/v) from 45% to over 60% in comparison with control cells in the absence of ethanol. Subsequent analysis of growth medium revealed that it contained approximately 0.35 mM magnesium ions.

Table 3. Metal ion specificity.

Treatment	% Control Activity	
	Phosphate Buffer	Growth Medium
Control	100	100
5% (w/v) EtOH-W	56	--
5% (w/v) EtOH-U	50	--
10% (w/v) EtOH-W	--	75
E-(W) + 0.5 mM Mg^{2+}	100	100
E-(W) + 1 mM Mg^{2+}	100	100
E-(W) + 0.5 mM Mn^{2+}	89	95
E-(W) + 1 mM Mn^{2+}	92	97
E-(W) + 0.5 mM Zn^{2+}	65	78
E-(W) + 1 mM Zn^{2+}	75	82
E-(W) + 0.5 mM Ca^{2+}	55	75
E-(W) + 1 mM Ca^{2+}	53	78
E-(W) + 2 mM Ca^{2+}	53	80
E-(W) + 5 mM Ca^{2+}	55	--
E-(W) + 0.5 mM Co	54	62
E-(W) + 1 mM Co	53	52
E-(W) + 2 mM Co	53	43
E-(W) + 5 mM Co	53	--

W = Washed cells after 10 min exposure to ethanol.

U = Unwashed cells; continuous presence of ethanol during the assay.

E (W) = 5% (w/v) ethanol in phosphate buffer assay (1.09 M) or 10% (w/v) in growth medium (minus glucose) assay (2.18 M).

EtOH = Ethanol

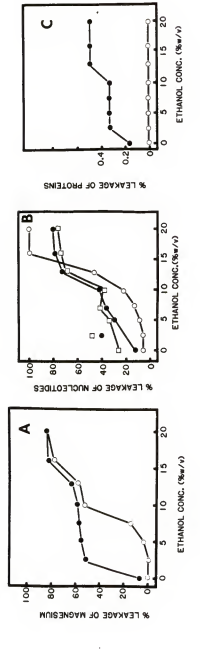
Ethanol-Induced Leakage

The effects of added ethanol on cellular leakage are shown in Figure 6. Cells were exposed to ethanol, centrifuged, and the supernatants examined to determine the extent of leakage. The 100% values obtained corresponded to 1.4 umoles of magnesium per mg of cell protein, 5.4 umoles of nucleotide (ADP-equivalents based upon absorbance at 260 nm) per mg of cell protein, and 70% of the total cellular protein. Increasing concentrations of ethanol caused a dose-dependent (though not linear) increase in the leakage of nucleotides and magnesium. The leakage of magnesium was more sensitive to the addition of ethanol than was nucleotide leakage. The addition of 1.1 M ethanol in buffer and 2.8 M ethanol (12.7% w/v) in growth medium resulted in the leakage of 50% of the magnesium. The leakage of 50% of the cellular nucleotides was caused by 2.4 M ethanol (11% w/v) in buffer and 2.8 M ethanol in growth medium. Appreciable leakage of cellular proteins was not observed even in the presence of 4.4 M ethanol.

Effect of Nucleotides Plus Magnesium Addition on the Inhibition of Fermentation by 4.4 M Ethanol

The damaging effects of low ethanol concentrations were almost completely reversed by the addition of magnesium ions but this addition was insufficient to prevent the inhibition of fermentation at high ethanol concentrations (3.3 and 4.4 M). Since both nucleotides and magnesium ions are required

Figure 6. Effects of ethanol on cell leakage. Cells were exposed to ethanol for 30 min at 30°C in either 50 mM phosphate buffer (pH 6.0) or growth medium, pelleted by centrifugation, and the extent of leakage of magnesium ions (A), of nucleotides (B), and of proteins (C) determined. Values are plotted as the percentage of constituent released by membrane disruption using chloroform (defined as 100%). Symbols for A and C: ● , buffer; ○ , growth medium. Symbols for B: □ , absorbance 260 nm in buffer; ● , ^{32}P-phosphate in buffer; ○ , ^{32}P-phosphate in growth medium.

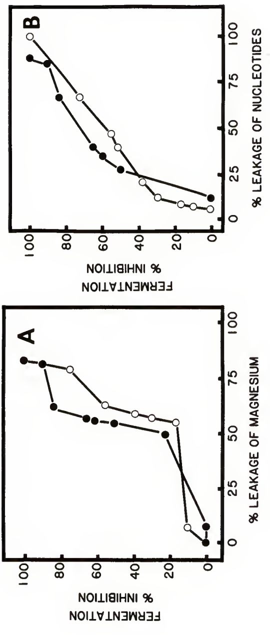

A
% LEAKAGE OF MAGNESIUM
0
20
40
60
80
100
ETHANOL CONC. (%w/v)
0
5
10
15
20
B
% LEAKAGE OF NUCLEOTIDES
0
20
40
60
80
100
ETHANOL CONC. (%w/v)
0
5
10
15
20
C
% LEAKAGE OF PROTEINS
0
0.2
0.4
ETHANOL CONC. (%w/v)
0
5
10
15
20

for fermentation and ethanol increased the leakage of both, we have examined the effect of nucleotides added with magnesium on cells in which fermentation was completely inhibited by 4.4 M ethanol. The addition of magnesium sulfate alone resulted in the recovery of 20% of the control activity. In combination with 0.2 mM NAD^+, a decrease in activity was observed. In combination with 0.2 mM NAD, 0.9 mM ATP, and 0.1 mM ADP, 30% of the control activity was recovered. High ADP/ATP ratios were less effective. These results indicated that the enzymes of glycolysis are not sufficiently inactive to prevent fermentation even in the presence of 4.4 M ethanol.

Relationship between Leakage and the Extent of Inhibition of Fermentation

Figure 7 shows the relationship between ethanol-induced leakage of magnesium and nucleotides and the ethanol-induced inhibition of fermentation. The relationships between the extent of leakage of magnesium and the extent of inhibition of fermentation in growth medium and in buffer are very similar although different concentrations of ethanol were required to cause an equivalent extent of leakage. The leakage of up to 50% of the cellular magnesium was accompanied by a modest inhibition of fermentation (< 20%). Further leakage resulted in a very rapid decline in the rate of fermentation. Complete release of magnesium was accompanied by complete inhibition of fermentation.

Figure 7. Relationship between the extent of ethanol-induced leakage and ethanol-induced inhibition of fermentation. The extent of inhibition of fermentation by various ethanol concentrations in either 50 mM sodium phosphate buffer (pH 6.0) or growth medium is plotted against the extent of leakage of magnesium ions (A) and the extent of leakage of nucleotides (B) which occurred at these ethanol concentrations, respectively. Symbols: ○ , growth medium; ● , buffer.


A
FERMENTATION
% INHIBITION
100
80
60
40
20
0
0
25
50
75
100
% LEAKAGE OF MAGNESIUM
B
FERMENTATION
% INHIBITION
100
80
60
40
20
0
0
25
50
75
100
% LEAKAGE OF NUCLEOTIDES

The relationships between nucleotide leakage and the inhibition of fermentation in growth medium and in buffer were also similar. Even low levels of nucleotide leakage were accompanied by a reduction in fermentative activity. Although different concentrations of ethanol were required to cause similar extents of nucleotide leakage in buffer and in growth medium, both were accompanied by an equal inhibition of fermentation.

Discussion

The accumulation of ethanol as an end product of fermentation is known to cause a progressive inhibition in the rate of sugar conversion into ethanol by *Z. mobilis* (Burrill et al., 1983; Laudrin and Goma, 1982) and by yeasts (Aiba et al., 1968; Moulin et al., 1984; Nagodawithana and Steinkraus, 1976; Navarro and Durand, 1978; Novak et al., 1981). This phenomenon is particularly important during commercial ethanol production and increases the time required to complete the conversion of sugar substrate into ethanol and limits the final concentration of ethanol achieved (Ingram and Buttke, 1984). Many studies have hypothesized that this inhibition is due to a direct action of ethanol on key enzymes of glycolysis and ethanol production involving feedback inhibition or enzyme inactivation (Beaven et al., 1982; Hoppner and Doelle, 1983; Laudrin and Goma, 1982; Moulin et al., 1984; Nagodawithana and Steinkraus, 1976). However, *in vitro* studies of these enzymes in *Z. mobilis* and in *S. cerevisiae* do not fully support this hypothesis (Millar et al., 1982).

Ethanol caused a dose-dependent inhibition of fermentation *in vivo* with measurable inhibition at concentrations as low as 0.4 M (2% w/v) while concentrations over 3.3 M (15% w/v) are required to inhibit enzymes *in vitro*; even higher concentrations are required to inactivate these enzymes (Millar et al., 1982; Moulin et al., 1984). One possible explanation which has been proposed to reconcile these differences is that the actual intracellular concentration of ethanol during active fermentation is several-fold higher than in the medium (Millar et al., 1982; Moulin et al., 1984; Nagodawithana and Steinkraus, 1976). However, our measurements and those of Laudrin and Goma (1982) indicate that *Z. mobilis* is freely permeable to ethanol and that intracellular concentrations of ethanol in *Z. mobilis* do not reach the high levels which are required to inactivate key enzymes *in vitro* (Millar et al., 1982). Certainly other aspects of the intracellular environment could potentially increase the sensitivity of these enzymes to ethanol *in vivo* and this possibility should not be eliminated.

The results of these investigations indicate that ethanol causes an inhibition of enzymes involved in alcohol production as previously proposed (Ingram and Buttke, 1984; Moulin et al., 1984) but provide evidence for a novel, indirect mechanism of action by ethanol. The inhibition of fermentation by ethanol appears to result from increased leakage through the plasma membrane, allowing the loss of cofactors and coenzymes. This conclusion is supported by the lack of complete reversability of inhibition by ethanol removal even after exposure to low concentrations of ethanol (less than 5% w/v in some cases), by ethanol-induced leakage of magnesium ions and nucleotides, by a magnesium ion requirement for the repair of ethanol-induced damage to fermentation (after removal of ethanol), and by the ability of added magnesium and added nucleotides to increase the rate of fermentation in the presence of ethanol. The extent of leakage of magnesium ions and of nucleotides correlated well with the extent of inhibition of fermentation under two different experimental assay conditions in which the sensitivity of fermentation to ethanol-inhibition was very different, i.e., cells resuspended in phosphate buffer and cells resuspended in growth medium.

It is likely that the leakage of magnesium ions (small molecules) and that of nucleotides (molecules of intermediate size) are only symptomatic of a more generalized increase in membrane permeability which may include many other ions, cofactors, and metabolites such as intermediates of glycolysis. The ability of magnesium to restore activity in the presence of low concentrations of ethanol suggests that magnesium ion leakage is the dominant effect under these conditions and is consistent with the leakage of magnesium ions induced by low ethanol concentrations. A variety of enzymes in Entner-Doudoroff glycolysis require magnesium as a cofactor including glucokinase, glucose-6-phosphate dehydrogenase, phosphoglycerate kinase, and enolase (Dawes et al., 1966; Gibbs and DeMoss, 1954; Millar et al., 1982; Rose and Rose, 1969; Sols et al., 1971; Stouthamer, 1978). The metal ion specificity for the protection and repair of ethanol damage to fermentation is similar to the metal ion specifity for these enzymes, i.e., magnesium and manganese supporting activity and the inability of calcium to substitute. Thus it is not surprising that the leakage of magnesium ions results in an inhibition of fermentation. Unlike S. cerevisiae, alcohol dehydrogenase in Z. mobilis does not appear to require magnesium (Hoppner and Doelle, 1983; Wills et al., 1981). Other metal cofactors which may be needed for fermentation include calcium, reported to activate pyruvate decarboxylase in Z. mobilis (Hoppner and Doelle, 1983), and potassium for pyruvate kinase (Sols et al., 1971). However, neither the addition of calcium nor of potassium reduced the ethanol-inhibition of fermentation.

Higher concentrations of ethanol were required to induce the leakage of nucleotides than were required for magnesium leakage, consistent with a requirement for more extensive membrane damage to allow leakage of these larger molecules. Magnesium alone only partially relieved the inhibition of fermentation under these conditions. At very high concentrations of ethanol (4.4 M) in which fermentation was completely inhibited, 30% of the fermentative activity of cells could be restored by the addition of nucleotides with magnesium ions. It is unlikely that fermentative activity can be completely restored in the presence of such high concentrations of ethanol due to the probable diffusion of intermediary metabolites out of and into the cell and due to a possible reduction in the catalytic rates of enzymes involved in glycolysis and alcohol production (Millar et al., 1982). However, these results demonstrate that the complete inhibition of fermentation by 4.4 M ethanol (20% w/v) does not result from feedback inhibition or enzyme denaturation even at these high concentrations of ethanol.

Previous studies with a variety of systems have shown that ethanol increases membrane leakage (Dombek et al., 1985; Eaton et al., 1982; Ingram and Buttke, 1984; Michaelis and Michaelis, 1983; Pang et al., 1979). There are three basic ways in which ethanol would be expected to decrease the effectiveness of the plasma membrane as a hydrophobic barrier: by altering the colligative properties of the environment, by directly interacting with the membrane, and by altering the dielectric properties of the environment. First, the addition of ethanol to an aqueous milieu is known to alter water structure and to decrease the strength of hydrophobic interactions (Yaacobi and Ben-Naim, 1974). This decreased strength of hydrophobic interactions would tend to decrease the extent to which acyl chains participate in hydrophobic interactions which form the hydrophobic core of the membrane and to increase the extent of incursion of polar molecules into the two membrane surfaces. High concentrations of ethanol would be expected to actually solubilize some of the membrane components by increasing the ability of the milieu to accommodate hydrophobic functional groups (Nozaki and Tanford, 1971; Tanford, 1978). In addition, high molar concentrations of ethanol would be expected to replace water to some extent as a hydrogen bonding partner and in solvation shells (hydration shell which includes ethanol). Secondly, ethanol is an amphipathic molecule and is known to partition into the hydrophobic region of the membrane (Dombek and Ingram, 1984; Goldstein et al., 1982; Rottenberg et al., 1981; Seeman et al., 1971). Since ethanol is much more polar than the hydrocarbon membrane core, its presence would tend to increase the average polarity of this environment and reduce its effectiveness as a hydrophobic barrier. The partitioning of ethanol into the membrane is also known to increase bulk membrane fluidity; increased fluidization per se has been shown to increase membrane leakage (Pang et al., 1979). Finally, the presence of ethanol at relatively high molar concentrations alters the dielectric properties of an aqueous milieu strengthening coloumbic interactions and shifting the equilibrium of charged ionic species toward the neutral, conjugated forms (Franks and Ives, 1966; Jukes and Schmidt, 1934). In general, membranes are more permeable toward these neutral species (Freeze et al., 1973). It is not possible to predict the biological consequences of these changes in coulombic interactions on membranes. However, such changes may alter the packing arrangement and allow segregation of membrane components such that discontinuities are produced which permeabilize cells, allowing the leakage of small molecules but retention of larger molecules. Such an effect is consistent with the release of cellular magnesium and nucleotides observed with 4.4 M ethanol in the absence of significant protein leakage. This type of global change in membrane organization is also consistent with the continued loss of fermentative activity in thanol-treated cells during subsequent incubation in buffer after ethanol removal.

Our initial experiments indicated that ethanol was more inhibitory for fermentation in phosphate buffer than in growth medium even though phosphate buffer was a component of growth medium. The basis of this difference in sensitivity appears to be the presence of magnesium ions in the yeast extract equivalent to 35 micromoles per gram. The addition of magnesium to phosphate buffer decreased the ethanol-inhibition of fermentation to a level equivalent to that of growth medium. The addition of EDTA to fermentation assays containing growth medium increased the extent of ethanol inhibition providing further evidence for the importance of divalent metals in the growth medium as the basis for the reduction in ethanol damage.

Microorganisms are known to actively transport magnesium (Jasper and Silver, 1977). An active transport system would be expected to partially counter the increased leakage of magnesium across the plasma membrane when appreciable quantities of magnesium ions are available in the surrounding milieu. Similarly, increasing the extracellular concentration of magnesium by addition would be expected to decrease the problem of providing sufficient intracellular magnesium for use as an enzyme cofactor. It is also likely that active magnesium transport is responsible for the repair of ethanol-damage to fermentation which occurs during incubation in fresh growth medium lacking ethanol. Thus the presence of extracellular magnesium in yeast extract is sufficient to explain the observed reduction in the potency of ethanol as an inhibitor of fermentation in growth medium as compared to buffer.

CHAPTER III
GLYCOLYTIC FLUX IN *Zymomonas mobilis*: ENZYME AND METABOLITE LEVELS DURING BATCH FERMENTATION

Introduction

Zymomonas mobilis is a unique bacterium which utilizes the Entner-Doudoroff glycolytic pathway for the fermentative production of ethanol and carbon dioxide from glucose (Montenecourt, 1985). This organism lacks an oxidative electron transport system and is obligately fermentative, producing a single net ATP per glucose metabolized. Glucose uptake by *Z. mobilis* is not energy requiring and proceeds via facilitated diffusion (Dimarco and Romano, 1985), although it is likely that other transport systems for magnesium, a nitrogen source, phosphate, etc, are energy requiring. Previous studies have provided evidence that glucose metabolism rather than uptake limits the rate of alcohol production in this organism (Doelle, 1982).

The rate of glycolysis and ethanol production by *Z. mobilis* (Moulin et al., 1984) like that of yeasts (Millar et al., 1982) declines progressively as ethanol accumulates in the surrounding medium during batch fermentation. This accumulation of ethanol has been proposed as the cause for the decrease in fermentative activity (Millar et al., 1982).

Although there is no satisfactory explanation for the progressive slowing of fermentation as ethanol accumulates, several hypotheses have been proposed including feedback inhibition or inactivation of glycolytic and alcohologenic enzymes (Hoppner and Doelle, 1983; Millar et al., 1982), ethanol-induced leakage of coenzymes and cofactors (Osman and Ingram, 1985), and nutritional limitations (Casey et al., 1984).

Key regulatory enzymes (prior to hexose cleavage) in Embden-Meyerhof glycolysis (Bosca and Corredor, 1984; Cooper, 1978) such as phosphofructokinase and an allosteric hexokinase are not present in the Entner-Doudoroff pathway in *Z. mobilis* (Montenecourt, 1985). However, the latter steps in the two pathways are similar (from glyceraldehyde-3-phosphate to pyruvate and ethanol). Thus it is possible that both differences and similarities may exist between yeasts and *Z. mobilis* which regulate glycolytic flux. *Z. mobilis* ferments glucose with 98% of theoretical efficiency, allowing the convenient and direct measurement of glycolytic flux as CO_2 evolved. In this study, we have attempted to identify possible constraints on glycolysis in *Z. mobilis* by comparing the levels of enzymes, cofactors, and metabolites with direct measurements of glycolytic flux (CO_2 evolved/mg cell protein per h), energy charge, internal pH, and ΔpH at different stages of batch fermentation. These components and a semipermeable membrane represent the essential requirements for fermentation.

Materials and Methods

Organism and Growth Conditions

Zymomonas mobilis strain CP4 was generously supplied by A. Ben-Bassat of Cetus Corporation (Berkeley, CA). This organism was grown at 30°C in the complex medium described by Skotnicki et al. (1981) with various levels of glucose. Stock cultures were maintained on solid medium containing 2% glucose and 1.5% agar. Fermentations (250 ml broth, 20% glucose) were carried out in "250-ml" spinner bottles designed for tissue culture (Bellco Glass, Inc., Vineland, NJ).

To prepare inocula for fermentation experiments, cells were transferred from solid medium to 18 x 150 mm culture tubes containing broth (10 ml, 0.2% glucose) and allowed to grow to an optical density (550 nm) of 0.8. Each fermentation bottle was inoculated with 5 ml of this suspension at zero times in fermentation experiments.

Samples were removed at various times for analysis. Glucose was measured using a YSI Industrial analyzer (Yellow Springs, OH). Protein was measured using the Folin reagent as described by Layne (1957). Ethanol was measured by gas chromatography as described by Goel and Pamment (1984) and is expressed as percentage weight per unit volume throughout this article.

Glycolytic flux was determined as fermentative activity by measuring the rate of CO_2 evolution at 30°C under a nitrogen atmosphere in a Gilson Differential Respirometer (Middleton, WI). Activities are expressed as umoles CO_2 evolved/mg cell protein per h.

Magnesium

Samples (1 to 3 ml) were removed at various times during fermentation and the cells harvested by centrifugation (10,000 x g, 2 min) using a microcentrifuge. Cells were washed once with 1 ml of sodium phosphate buffer (50 mmolar, pH 6.0). The resulting cell pellets were resuspended in 0.2 ml of this buffer and permeabilized by mixing with 2 drops of chloroform on a vortex mixer. Magnesium concentrations were determined in supernatants from the permeabilized cell preparations using the "60-Second Magnesium" assay supplied by the American Monitor Corporation (Indianapolis, IN).

Adenine and Nicotinamide Nucleotides

Nucleotides were extracted by mixing 0.3 ml of culture (from various times during fermentation) with 0.7 ml of cold absolute ethanol as described by Karp et al. (1983). Cell debris was removed by centrifugation (10,000 x g, 2 min) and the samples immediately assayed for either adenine or nicotinamide nucleotides using luminescence assays (Strehler, 1968) and a Beckman Model 8000 scintillation counter (Fullerton, CA) with the coincidence switched off.

The adenine nucleotides; ATP, ADP, and AMP; were measured using the firefly luciferase assay system supplied by LKB Wallac (Gaithersburg, MD). The AMP was converted to ADP using adenylate kinase and ADP to ATP using pyruvate kinase essentially as described by Chapman et al. (1971).

The $NADP^+$ and NADPH were determined using the bacterial (Beneckea harveyi) luciferase reagents supplied by LKB Wallac (Gaithersburg, MD). The $NADP^+$ was converted to NADPH using glucose-6-phosphate dehydrogenase. The NAD^+ and NADH were determined using bacterial luciferase assay reagents (Picorase D) obtained from Packard Instrument Company (Downers Grove, IL). The NAD^+ was converted to NADH using alcohol dehydrogenase.

Internal pH and ΔpH

Internal pH and ΔpH were measured as described by Kashket (1981) using [7-^{14}C]-benzoic acid (New England Nuclear, Boston, MA). Measurements of $\Delta\psi$ were also attempted with [U-^{3}H]-tetraphenylphosphonium bromide (Amersham Corp., Arlington Heights, IL) but none was detected.

Permeabilization of Cells for Enzyme Assays

Cells (1 to 2 ml) were harvested from various times during fermentation by centrifugation (10,000 x g, 2 min) and washed once in 50 mmolar sodium phosphate buffer (pH

6.0). These were resuspended in 0.2 ml of buffer and permeabilized by mixing with 2 drops of chloroform for 15 sec using a vortex mixer followed by the addition of 0.8 ml of cold buffer. Permeabilized cells were held on ice and appropriate dilutions of this suspension were assayed immediately for individual enzymes. No more than 2 enzymes were measured during each set of fermentation experiments.

Cell Lysates

Cells were rapidly chilled and harvested at various times during batch fermentation by centrifugation (6,000 x g, 5 min) using a Sorvall RC 2B centrifuge. Approximately 2 g of fresh cell pellet (wet weight) was resuspended in 4 ml of 50 mmolar sodium phosphate buffer (pH 6.0) and passed through a cold French Pressure cell twice at 20,000 psi (three passages for the stationary phase cells). Unbroken cells and debris were removed by centrifugation at 6,000 x g for 5 min ($4^{o}C$). The supernatant was held on ice and appropriate dilutions assayed immediately for enzymatic activities.

Enzyme Assays

The specific activities of the Entner-Doudoroff pathway enzymes and the alcohologenic enzymes were determined in permeabilized cells and in cell lysates under substrate saturating conditions. Activities were measured spectrophotometrically at ambient temperature (23°C) by following the oxidation or reduction of nicotinamide nucleotides directly or in coupled assay systems using a Beckman Model 25 kinetic spectrophotometer (Fullerton, CA). Reactions were begun by the addition of substrate. All substrates and coupling enzymes were obtained from the Sigma Chemical Co. (St. Louis, MO).

Glucokinase (EC 2.7.1.12) and glucose-6-phosphate dehydrogenase (EC 1.1.1.43) were assayed as described by Hylemon and Phibbs (1972). The combined activities of 6-phosphogluconate dehydratase (EC 4.2.1.12), 6-phosphogluconolactonase (EC 3.1.1.31) and 2-keto-3-deoxy-6-phosphogluconate aldolase (EC 4.1.2.14) were assayed as described by Lessie and Neidhardt (1967) by coupling pyruvate formation to lactate production with lactate dehydrogenase. Glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13) was assayed as described by Byers (1982) using the arsenolysis reaction. The activity of 3-phosphoglycerate kinase (EC 2.7.2.6) was measured by coupling to glyceraldehyde-3-phosphate dehydrogenase as described by Maitra and Lobo (1971a). Phosphoglycerate mutase (EC 2.7.5.3) was measured by coupling with enolase, pyruvate kinase, and lactate dehydrogenase (Marita and Lobo, 1971a). Enolase (EC 4.2.1.11) was assayed using the same system described for phosphoglycerate mutase but with mutase as a coupling enzyme instead of enolase (Marita and Lobo, 1971a). Pyruvate kinase (EC 2.7.1.40) was assayed as described by Ng and Hamilton (1975) by coupling with lactate dehydrogenase. Pyruvate decarboxylase (EC 4.1.1.1) and alcohol dehydrogenase (EC 1.1.1.1) were assayed as described by Hoppner and Doelle (1983).

NMR Comparison of Phosphorylated Metabolites

Cells were grown in batch fermentations as previously described and harvested by centrifugation at ambient temperature (6,000 x g, 5 min). The resulting cell pellet was resuspended in 1/20 volume of fresh growth medium or washed with 100 ml of fermentation buffer (100 mM MES-HCl, 74 mmolar potassium phosphate, 50 mmolar magnesium chloride, and 20% glucose, pH 6.0) and resuspended in 1/20 volume of fermentation buffer. Cells in fermentation buffer or in fresh growth medium were incubated at 30^{o}C with agitation until the glucose concentration had fallen to 15% and were rapidly inactivated by the addition of 0.2 volumes of cold perchloric acid (30%). Samples were held on ice with periodic warming to room temperature to facilitate extraction. Cellular debris was removed by centrifugation (15,000 x g, 15 min, 0^{o}C) and the supernatant neutralized with 2 molar potassium bicarbonate. The precipitate was removed by centrifugation and the supernatant stored frozen at -70^{o}C. Samples were subjected to several freeze/thaw/centrifugation cycles to remove additional potassium perchlorate prior to adding deuterium oxide and solid sodium ethylene diamine tetraacetic acid (15% and 30 mM final concentrations, respectively) and adjusting to pH 8.2 as described by Barrow et al. (1984).

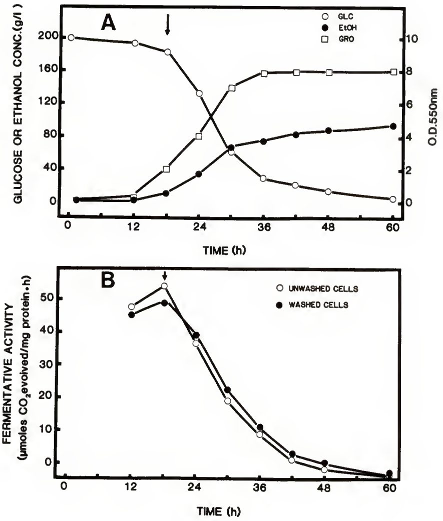
Phosphorus-31 NMR was performed using a Nicolet NT-300 multinuclear tunable Fourier transform spectrometer at 70.5 kG magnetic field strength. All samples were examined in the Fourier transform mode with broad band decoupling of protons at ambient temperature (23°C). Spectra were taken at a frequency of 121.5 MHz (spectral width, 6024 Hz; pulse width, 42.5 us; acquisition time, 0.680 s) and 10,000 scans accumulated for each sample. Chemical shifts are expressed relative to 85% orthophosphoric acid but were measured relative to hexachlorcyclotriphosphorazene (17.4 mg/ml HCCTP with 0.5 mg/ml chromium triacetate to speed relaxation) in a sealed capillary as suggested by Gard and Ackerman (1983). This internal standard was included during the accumulation of spectra and results are scaled to reflect equivalence of this standard. Identifications were based upon comparison to spectra reported by Barrow et al. (1984).

Results

Changes in Fermentative Activity of Cells During Batch Fermentation

Figure 8A shows an average of three batch fermentations with *Z. mobilis* strain CP4 (20% glucose). Exponential growth (2 h generation time) ended after 18 h although cell mass (optical density at 550 nm) continued to increase rapidly for the first 30 h. Growth slowed dramatically

Figure 8. Fermentation of 20% glucose by *Z. mobilis* strain CP4. A. Growth □ , glucose ○ consumption, and ethanol ● production. B. Fermentative activity of cells from various times during fermentation. Cells measured directly in fermentation broth, ○ ; cells resuspended in fresh medium lacking ethanol, ●. The arrow indicates time of maximum fermentative activity.


A
○ GLC
● EtOH
□ GRO
GLUCOSE OR ETHANOL CONC.(g/l)
0
40
80
120
160
200
O.D.550nm
0
2
4
6
8
10
0
12
24
36
48
60
TIME (h)
B
○ UNWASHED CELLS
● WASHED CELLS
FERMENTATIVE ACTIVITY
(μmoles CO2 evolved/mg protein·h)
0
10
20
30
40
50
0
12
24
36
48
60
TIME (h)

after 30 h reaching stationary phase before 36 h. Glucose consumption and ethanol production also slowed dramatically after 30 h with a final yield of 9.8% ethanol (98% of theoretical yield) after 60 h. Although it is not evident from this fermentation profile, the fermentative activity of the cells changed dramatically as the batch fermentation proceeded.

Fermentative activity or glycolytic flux was measured by determining the rate of CO_2 evolution per mg cell protein at various times during fermentation (Fig. 8B). The highest activity was observed at 18 h (1.1% accumulated ethanol) and declined to 1/2 of the maximal activity after 30 h (6.2% accumulated ethanol). Little activity was evident after 60 h (9.8% ethanol), presumably due to the exhaustion of glucose. A solid arrow has been placed at the peak of fermentative activity and is also included on subsequent figures as a point of reference.

To examine the possibility that the presence of ethanol or other end products of growth and fermentation were directly responsible for the inhibition of fermentative activity, samples were removed, washed once in fresh growth medium, and resuspended in fresh growth medium (20% glucose) lacking ethanol (Fig. 8B). Removal of ethanol and fermentation products did not increase fermentative activity. Thus the decline in fermentative activity was not reversed by the removal of ethanol or other fermentation products or by restoring the initial glucose concentration.

This decline appears to reflect physiological changes in the cells which are not immediately reversible.

Cell death represents one possible explanation for the progressive decline in fermentative activity. To examine this possibility, samples were diluted and spread on solid medium to determine viable cell number (colony forming units) at various times during batch fermentation (Fig. 9). Viable cell number continued to increase rapidly for 30 h, well beyond the onset of the decline in fermentative activity (arrow). The maximal cell number was reached after 30 h, declining slowly for the following 18 h and abruptly falling after 48 h with a 99% loss of viability. Thus the initial decline in fermentative activity does not appear to result from a decrease in the number of viable cells, although this may be an important factor during the latter stages of batch fermentation.

Magnesium

Magnesium is required as a counter ion for the nucleotides used by many glycolytic enzymes. In a previous study, we have shown that the leakage of magnesium is responsible in part for the decrease in fermentative activity caused by the addition of high concentrations of ethanol (Osman and Ingram, 1985). Figure 10A shows the cellular levels of magnesium during batch fermentation. The initial magnesium concentration in fresh growth medium was 25 umolar. Cellular magnesium content increased during the

Figure 9. Changes in viable cell number (colony forming units, ○) and cell protein (mg/ml, ●) broth during batch fermentation. The arrow indicates time of maximal fermentative activity.

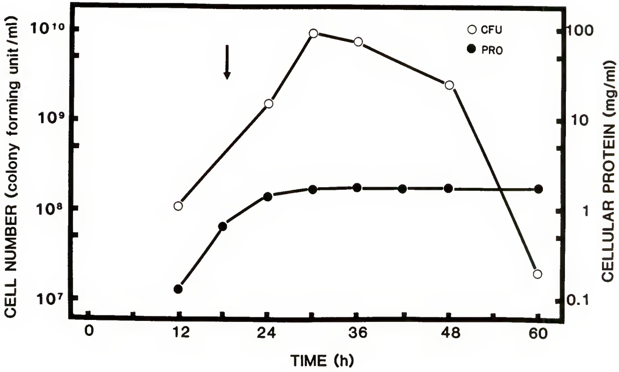
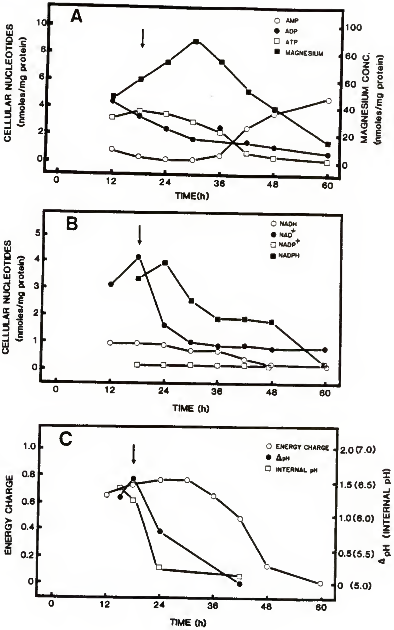

CELL NUMBER (colony forming unit/ml)
10^{10}
10^9
10^8
10^7
CFU
PRO
100
10
1
0.1
CELLULAR PROTEIN (mg/ml)
0
12
24
36
48
60
TIME (h)

Figure 10. Changes in nucleotides, cellular magnesium, energy charge, and pH during batch fermentation. A. Adenine nucleotides and magnesium. Symbols: □ , ATP; ● , ADP; ○ , AMP; magnesium, ■. B. Nicotinamide nucleotides. Symbols: ● , NAD^+; ○ , NADH; □ , $NADP^+$; ■ , NADPH. C. Energy charge ○, ΔpH ●, and internal pH □. The arrow indicates time of maximal fermentative activity.


A
○ AMP
● ADP
□ ATP
■ MAGNESIUM
CELLULAR NUCLEOTIDES (nmoles/mg protein)
0
2
4
6
8
10
MAGNESIUM CONC. (nmoles/mg protein)
0
20
40
60
80
100
0
12
24
36
48
60
TIME(h)
B
○ NADH
● NAD$^+$
□ NADP$^+$
■ NADPH
CELLULAR NUCLEOTIDES (nmoles/mg protein)
0
1
2
3
4
5
0
12
24
36
48
60
TIME (h)
C
○ ENERGY CHARGE
● ΔpH
□ INTERNAL pH
ENERGY CHARGE
0
0.2
0.4
0.6
0.8
1.0
ΔpH (INTERNAL pH)
0 (5.0)
0.5(5.5)
1.0(6.0)
1.5 (6.5)
2.0 (7.0)
0
12
24
36
48
60
TIME (h)

initial stages of fermentation, reaching a maximum of 90 nmoles/mg cell protein after 30 h, and then declining to 12 nmolar at 60 h. Assuming an intracellular volume of 2.4 uliters/mg cell protein (Osman and Ingram, 1985), the magnesium concentration during the highest rate of fermentation (18 h, 58 nmoles/mg cell protein) corresponds to an intracellular level of approximately 24 nmolar. Since the intracellular content of magnesium continued to increase well beyond the peak in fermentative activity (solid arrow), a magnesium deficiency does not appear to be involved in the early decline in the rate of fermentation.

Nucleotides

The levels of the individual adenine nucleotides were measured during fermentation (Fig. 10A). Cells were inactivated in broth and the combined levels within the cell and any nucleotides which had leaked into the medium were determined. As a control for possible nucleotide leakage, cells were pelleted and the supernatants examined for the levels of adenine nucleotides. No detectable AMP, ADP, or ATP were found in broth from fermentation times shorter than 24 h. At 30 h, 1% to 2% of the total adenine nucleotides were present in the broth. After 42 h, 4%-6% of the total nucleotides were present in the broth and 6%-8% of the total appeared in the broth after 48 h. The adenine nucleotides present in broth were not limited to a particular form of adenine and all were present at roughly equal levels, relative to that of the combined measurement (broth plus cells). Although the nicotinamide nucleotides were not measured in broth, it is likely that they were retained to the same extent or greater since they are somewhat larger in size than the adenine nucleotides. The retention of adenine nucleotides indicates that membrane integrity was preserved in large part during batch fermentation, although it is possible that smaller molecules may have been lost. The level of ATP remained relatively constant over the first 30 h with a peak of 3.6 nmoles/mg cell protein after 18 h. This level declined rapidly after 30 h reaching 0.2 nmoles/mg cell protein at the end of fermentation. In contrast, the level of ADP was highest at the earliest point measured, 12 h, and appeared to decline during the remaining fermentation. The level of AMP was initially low and continued to decline with a minimum at 24 h. The subsequent increases in the levels of AMP mirrored the decreases of ATP reaching a maximum of 4.7 nmoles/mg cell protein after 60 h. Assuming an intracellular volume of 2.4 uliters/mg cell protein, maximal concentrations of AMP, ADP, and ATP were 1.5 to 2.0 mM. At 18 h, the peak in fermentative activity, the ATP/ADP ratio was near 1.

The levels of NAD^+ and NADH were also examined (Fig. 10B). The NAD^+ peaked after 18 h (4.2 nmoles/mg cell protein) and declined rapidly to 1/4 this level after 30 h. The level of NADH was much lower initially and continued to decline during fermentation. The NAD^+/NADH ratio was approximately 4 at 18 h, the peak of fermentative activity, and declined to near 1 after 30 h. At the peak of fermentative activity, the approximate intracellular concentrations of NAD^+ and NADH were 1.8 and 0.4 mmolar, respectively.

During batch fermentation, the levels of NADPH remained higher than those of $NADP^+$ in contrast to the NAD^+/NADH ratios (Fig. 10B). The NADPH levels were high at 18 h and 24 h (3.3 and 3.9 nmoles/mg cell protein), decreasing to approximately 0.1 nmoles/mg cell protein after 60 h. At 18 h, the peak of fermentative activity, the intracellular concentrations of $NADP^+$ and NADPH were approximately 0.4 mmolar and 1.4 mmolar, respectively.

The peak of fermentative activity and subsequent decline roughly paralleled the changes in the level of NAD^+, the NAD^+/NADH ratio, and the total nicotinamide nucleotides. No other measured changes in nucleotide levels or ratios exhibited a similar trend.

Energy Charge, ΔpH, and Internal pH

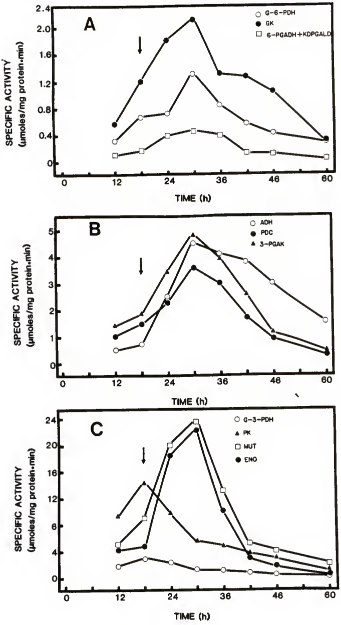
The energy charge of cells from different stages of fermentation was calculated from the measurements of adenine nucleotides and is plotted in Fig. 10C. Like ATP, overall energy charge was relatively high during the initial stage of fermentation reaching 0.8 between 24 h and 30 h. Energy charge declined rapidly after 30 h falling to near zero at the end of fermentation (60 h). In contrast, the ΔpH of cells in native fermentation broth exhibited a sharp peak at 18 h, the time at which fermentative activity was at its maximum (solid arrow) and fell rapidly to near zero by the end of 42 h (8% accumulated ethanol), coincident with the loss of colony forming ability (Fig. 9). An ethanol concentration of 8% has previously been shown to prevent the growth of Z. mobilis (Dombek et al., 1985; Ingram, 1986). Thus the observed decline in ΔpH during fermentation is consistent with an ethanol-induced leakage of hydrogen ions into cells in excess of excretion by metabolic pumps.

Internal pH (Fig. 10C) followed a trend similar to that of fermentative activity (Fig. 8B). Internal pH was initially high, pH 6.5 at 12 h and pH 6.3 at 18 h, declining to 5.4 after 24 h and to 5.1 after 42 h. During this period, the pH of the fermentation broth declined from pH 5.2 at 12 h to pH 4.6 at 24 h and increased to pH 5.1 after 42 h.

Enzyme Levels During Batch Fermentation

The specific activities of most of the enzymes which convert glucose to ethanol and CO_2 were measured in permeabilized cells taken from various stages of fermentation under substrate-saturating conditions (Fig. 11). Nine enzymes were examined individually; the combined activities of enzymes from 6-phosphogluconate through the cleavage of 2-keto-3-deoxy-6-phosphogluconate (KDGP) were measured together. The specific activities of all of these

Figure 11. Changes in the *in vitro* activities (umoles/mg cell protein per min) of glycolytic and alcohologenic enzymes during batch fermentation. A. Glucokinase, ●; glucose-6-phosphate dehydrogenase, ○; and the combined activities of 6-phosphogluconate dehydratase, 6-phosphogluconolactonase, and KDGP aldolase, □. B. Alcohol dehydrogenase, ○; pyruvate decarboxylase, ●; and 3-phosphoglycerate kinase, ▲. C. Pyruvate kinase, ▲; phosphoglycerate mutase, □; enolase, ●; and glyceraldehyde-3-phosphate dehydrogenase, ○. The arrow indicates time of maximal fermentative activity.


A
○ G-6-PDH
● GK
□ 6-PGADH+KDPGALD
SPECIFIC ACTIVITY
(µmoles/mg protein.min)
2.4
2.0
1.6
1.2
0.8
0.4
0
0
12
24
36
46
60
TIME (h)
B
○ ADH
● PDC
▲ 3-PGAK
SPECIFIC ACTIVITY
(µmoles/mg protein.min)
5
4
3
2
1
0
0
12
24
36
46
60
TIME (h)
C
○ G-3-PDH
▲ PK
□ MUT
● ENO
SPECIFIC ACTIVITY
(µmoles/mg protein.min)
24
20
16
12
6
4
0
0
12
24
36
46
60
TIME (h)

enzymes increased during the early stages of fermentation indicating that they were being synthesized more rapidly than bulk protein. Increases in specific activity between 12 h and 30 h were approximately 5-fold for most enzymes although smaller increases were observed for pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase. Since the fermentative rate expressed as umole of CO_2 evolved/mg cell protein per h represents the equivalent of 1/60 international enzyme unit (IU), these can be readily compared. Thus at 18 h, the point at which the maximum was observed, a minimum of 0.5 IU is required for glycolytic enzymes and 1.0 IU for alcohol dehydrogenase and pyruvate decarboxylase. This is roughly equivalent to the measured level of alcohol dehydrogenase and considerably higher than our estimate of the combined activities of 6-phosphogluconate dehydratase through the cleavage of KDGP. The reason for our low apparent activity in the combined enzyme assay is unclear but may be due in part to the previously noted instability of 6-phosphogluconate dehydratase (Scopes and Griffiths-Smith, 1984). All other enzymes were in excess of that required to support the measured levels of fermentative activity throughout batch fermentation.

The changes in the specific activities of these enzymes followed either of two patterns during the course of fermentation and are assigned as groups I and II. Group I enzymes (glucokinase, glucose-6-phosphate dehydrogenase, the combined activities of 6-phosphogluconate dehydratase through KDGP aldolase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate decarboxylase, and alcohol dehydrogenase) all increased in specific activity during the initial stages of fermentation reaching their highest specific activity at 30 h. Since the peak in specific activities of group I enzymes occurred well beyond the time at which fermentative activity began to decline, it is unlikely that the levels of these enzymes contribute to the observed early decline in fermentative activity.

Group II enzymes consisted of pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase (Fig. 11C). These enzymes exhibited a maximal specific activity at 18 h, coincident with the peak of fermentative activity (solid arrow) and declined thereafter. Indeed, a plot of the specific activities of these group II enzymes as a function of fermentative activity exhibits a linear relationship with the exception of the 12 h point (Fig. 12). Alcohol dehydrogenase is included as an example of a group I enzyme for comparison.

By plotting these data as activity per ml of fermentation broth, several additional features are apparent (Fig. 13). 3-Phosphoglycerate kinase and pyruvate kinase are shown as examples of group I and group II enzymes, respectively. The highest volumetric rate of fermentation (50 umoles of CO_2 evolved per ml of culture) was observed at 24 h, coincident with the maximal activity of group II enzymes. The activities of group I enzymes (Fig. 11)

Figure 12. Correlation between selected enzymes and fermentative activity. The *in vitro* activities (umoles/mg cell protein per min) from various stages in fermentation were plotted as a function of fermentation rate (umoles CO_2 evolved/h per mg cell protein). Symbols: pyruvate kinase (left axis), ●; glyceraldehyde-3-phosphate dehydrogenase (right axis), ○; alcohol dehydrogenase (left axis), □. The arrow indicates time of maximal fermentative activity.

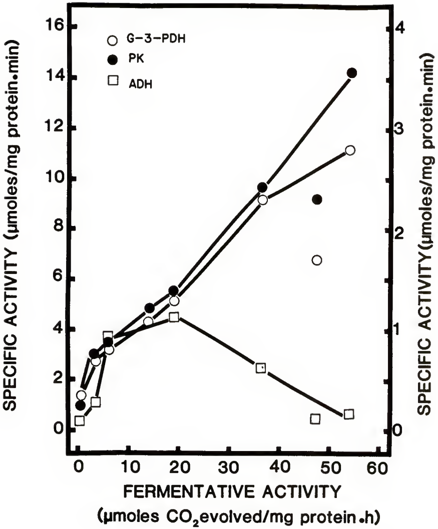
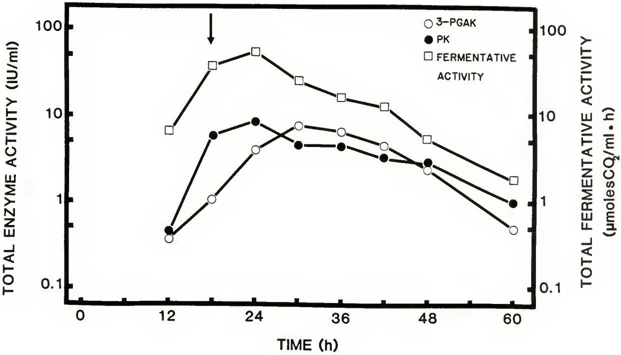

○ G-3-PDH
● PK
□ ADH
SPECIFIC ACTIVITY (µmoles/mg protein.min)
0
2
4
6
8
10
12
14
16
SPECIFIC ACTIVITY(µmoles/mg protein.min)
0
1
2
3
4
0
10
20
30
40
50
60
FERMENTATIVE ACTIVITY
(µmoles CO₂evolved/mg protein.h)

Figure 13. Changes in total enzyme (IU per ml broth) and volumetric productivity (umoles CO_2/h per ml broth) during batch fermentation. Symbols: pyruvate kinase, ●; 3-phosphoglycerate kinase, ○; volumetric productivity, □. The arrow indicates time of maximal fermentative activity.


TOTAL ENZYME ACTIVITY (IU/ml)
100
10
1
0.1
○ 3-PGAK
● PK
□ FERMENTATIVE ACTIVITY
TOTAL FERMENTATIVE ACTIVITY (µmolesCO2/ml • h)
100
10
1
0.1
0
12
24
36
48
60
TIME (h)

increased until 30 h, near the end of net protein synthesis. After reaching a maximum, the activities of both group I and group II enzymes declined slowly throughout the remaining fermentation.

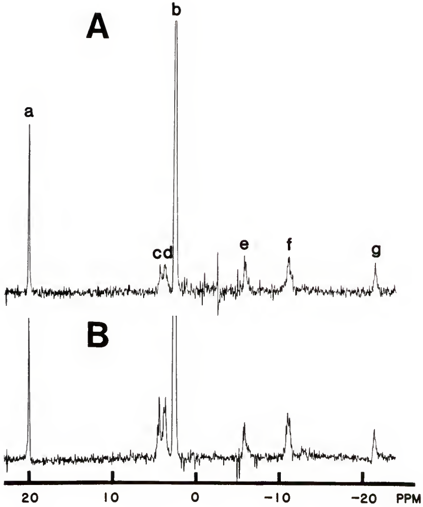
Total cell protein/ml has been included on Figure 2 for comparison. Exponential protein synthesis ended at approximately 18 h followed by a transition phase with no net synthesis after 36 h. Thus the initial decline in the specific activity of group II enzymes (Fig. 11C) appears to result primarily from continued synthesis of other proteins (Fig. 13) rather than inactivation. Bulk cellular protein was remarkably stable and did not decrease during the latter stages of fermentation (Fig. 9). Since the glycolytic enzymes represent a major portion of the total cellular protein, the decline in the measured activities and specific activities must represent limited inactivation rather than extensive proteolysis.

French press lysates of cells from 18 h, 30 h, and 48 h were examined to determine if the changes in specific activities of group I and II enzymes were due to the method of preparation. These results were in excellent agreement with the permeabilized cells. The group II enzymes (pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase) were both highest in the 18 h preparation and the group I enzymes (glucokinase and alcohol dehydrogenase) were highest in the 30 h preparation. The four enzymes examined were all lowest in the 48 h preparation.

NMR Comparison of Metabolite Levels

We have used ^{31}P-NMR to compare the relative abundance of nucleotides and metabolites in cells of *Z. mobilis* taken from two stages of batch fermentation, 18 h (the peak of fermentative activity per mg cell protein; 1.1% accumulated ethanol), and 30 h (1/2 maximal cellular rate of fermentation; 6.2% accumulated ethanol). This comparison was performed in two ways. Metabolites were compared in cells after washing and resuspending in MES buffer with glucose, magnesium, and phosphate (Fig. 14). Cells resuspended in MES buffer evolved CO_2 at roughly half the rate observed in original growth medium. Two relatively sharp peaks were observed in all spectra and represent the internal standard, HCCTP, and inorganic phosphate. These are labelled a and b, respectively. In MES buffer, five clusters of peaks were observed in both 18 h and 30 h cells, labelled c through g. Based upon the identification of Barrow et al. (1984), peak cluster c corresponds to glyceraldehyde-3-phosphate, 3-phosphoglyceric acid, KDGP and 6-phosphogluconate. Peak cluster d corresponds to a mixture of sugar mono-phosphates and nucleotide monophosphates; peak cluster e corresponds to ATP-γ, UTP-γ, ADP-β, and UDP-β; peak cluster f corresponds to α-phosphates in adenine and uridine nucleotides, oxidized nicotinamide nucleotides, and UDP-sugars; peak cluster g corresponds to the ATP-β and UTP-β. The size relationships among these clusters of peaks did not reveal any measurable differences between 18 h and

Figure 14. Phosphorus-31 NMR spectra of perchlorate extracts from cells taken from different stages of fermentation and incubated in MES buffer. A. Cells removed from fermentation after 18 h. B. Cells removed from fermentation after 30 h. Letters are defined in text.

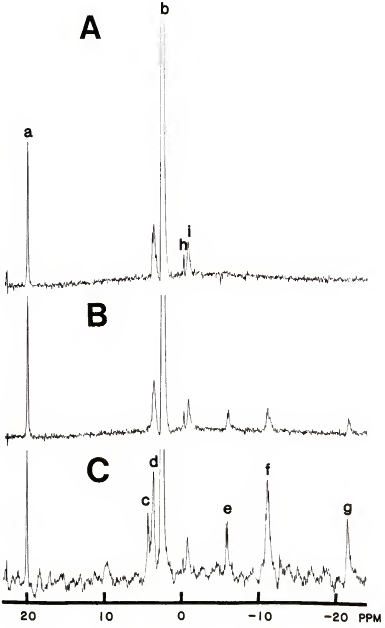

A
B
a
b
c
d
e
f
g
20
10
0
-10
-20
PPM

30 h cells in MES buffer. The spectra of these cell preparations are in good agreement with the results of Barrow et al. (1984) with Z. mobilis using a similar buffer/reaction system.

Experiments in growth medium instead of buffer were somewhat more complicated (Fig. 15). Unused, autoclaved growth medium contained substantial amounts of the components of peak cluster d and two new peaks, h and i (unidentified), in addition to inorganic phosphate. These components were present in all extracts of cells resuspended in growth medium and are presumed to be components of the autoclaved yeast extract. The other peaks observed in growth medium were similar to those in MES buffer except for relative intensities. Cells from 18 h were almost devoid of the glycolytic intermediates of peak cluster c although other clusters of peaks were reasonably prominent. In addition, 30 h cells in growth medium appeared to have reduced amounts of this peak cluster relative to other peak clusters when compared to 30 h cells in MES buffer. The relative intensity of peak cluster f was highest in the spectrum from 30 h cells in growth medium (mixture of nucleotide resonances).

These results demonstrated that very low levels of the glycolytic intermediates contained within peak cluster c are present during the most active stage of batch fermentation, 18 h cells in growth medium. The levels of these components increased when fermentation was impeded by resuspension in

Figure 15. Phosphorus-31 NMR spectra of perchlorate extracts from cell taken from different stages of fermentation and incubated in fresh fermentation broth. A. Broth control without cells. B. Cells removed from fermentation after 18 h. C. Cells removed from fermentation after 30 h.


A
B
C
a
b
c
d
e
f
g
h
i
20
10
0
-10
-20
PPM

MES buffer and in cells from an older stage of fermentation (30 h, in growth medium), consistent with a restriction in glycolysis at a later step in the process.

Discussion

Glycolytic flux, or the rate at which cells of *Z. mobilis* convert glucose to ethanol plus CO_2, does not remain constant during the batch fermentation of 20% glucose. The highest rate, approximately 0.5 umole glucose/min per mg cell protein, was achieved after 18 h with a 50% decline after 30 h. This initial decline in activity does not appear to result from cell death or to be relieved by the removal of ethanol and addition of fresh medium. Thus this initial decline in fermentation rate is apparently due to physiological changes within the cells. Viable cell number begins to decline after 30 h and cell death may be a major factor in the subsequent further decline in fermentative activity. Indeed, the decline in fermentative activity during the progressive conversion of glucose to ethanol may well reflect the combined effects of many detrimental physiological changes.

We have examined many of the factors involved in glycolysis and alcohol production including cellular magnesium, coenzymes, enzymes, and metabolites. Phosphorylated intermediates of glycolysis were more abundant in 30 h cells and in 18 h cells metabolizing under suboptimal fermentation conditions (buffer) than they were in optimally active cells at 18 h (growth medium), suggesting a limitation in glycolysis. The specific activities of ten of the enzymes examined (seven individually assayed and the combined activities of three) increased between 18 h and 30 h making it unlikely that a limitation in the amount of these enzymes contributed to the initial decline in glycolytic flux. Cellular magnesium, required as a cofactor for many of the glycolytic enzymes, also continued to increase. Adenylate charge remained high for the initial 30 h of fermentation.

At least three types of changes were observed in *Z. mobilis* during batch fermentation on 20% glucose between 18 h and 30 h, each of which could be responsible for a decline in the rate of glycolysis: reduced abundance of coenzymes, reduction in intracellular pH, and a reduction in the specific activities of two glycolytic enzymes. Total nicotinamide nucleotides (particularly NAD^+) declined by approximately 50% during the period from 18 h to 30 h. This decline would certainly be expected to reduce the rate of glycolysis and alcohol production since these enzymes typically function below substrate saturation *in vivo* (Algar and Scopes, 1985; Barrow et al., 1984) and to shift the equilibrium constants for dehydrogenase reactions. Total adenosine nucleotides also decreased but to a lesser extent. This decrease in total adenosine nucleotides was accompanied by an increase in the ATP/ADP ratio from approximately 1.0 at 18 h to 1.7 at 30 h, changes which may reduce the rates of glucose-6-phosphate dehydrogenase (Lessmann et al., 1975), glucokinase (Algar and Scopes, 1985), and pyruvate kinase (Ng and Hamilton, 1975; Smart and Pritchard, 1982; Thompson and Torchia, 1984). Intracellular pH decreased from pH 6.2 at 18 h to approximately pH 5.3 (interpolated from Fig. 3C) accompanied by a similar sharp decline in ΔpH. The decline in ΔpH in _Z_. _mobilis_ is similar to the dissipation of the proton motive force in clostridia by high levels of butanol (Gottwald and Gottschalk, 1985; Huang et al., 1985 and 1986). Since pH 5.3 is far below the optimum pH for most of the enzymes involved (Algar and Scopes, 1985), this change would be expected to reduce the rate of catalysis in _Z_. _mobilis_. The _in vitro_ specific activities of two of the enzymes examined, glyceraldehyde-3-phosphate dehydrogenase and pyruvate kinase, decreased by roughly 50% between 18 h and 30 h. Although the measured activities of these two enzymes under substrate-saturating conditions were in apparent excess of that required for fermentative activity, the specific activities of these two enzymes showed excellent correlation with the rate of glycolytic flux measured as the fermentative production of CO_2. Further, the total activities of these two enzymes per ml of culture also exhibited excellent correlation with the volumetric productivity of ethanol plus CO_2 which peaked at 24 h.

Previous studies in our laboratory (Dombek et al., 1985; Ingram and Buttke, 1984; Ingram, 1986; Osman and

Ingram, 1985) have emphasized the detrimental effects of ethanol on the plasma membrane, increasing the rate of leakage of small molecules. Ethanol has previously been reported to act as an uncoupler of oxidative phosphorylation by increasing hydrogen ion leakage in E. coli (Enequist et al., 1981). Thus the accumulation of ethanol may be responsible for the progressive collapse in pH (net leakage in excess of metabolic pumps) resulting in the acidification of the cytoplasm to a level which reduces glycolytic flux. Recent studies by Algar and Scopes (1985) utilizing a partially purified preparation of enzymes for glycolysis and alcohol production in vitro have provided further support for the detrimental effect of pH on overall glycolytic flux. They have shown that the rate of glycolysis and alcohol production remained highest when the pH was not allowed to fall below 6.5, the intracellular pH which we measured in cells at their peak in fermentative activity (18 h). Using this same system, these authors also showed that a balance of ATPase activity and glucose input was necessary for optimal fermentation to allow regeneration of inorganic phosphate and ADP. It seems unlikely that passive ethanol-induced leakage of nucleotides is an important factor for the decline in fermentative activity between 18 h and 30 h since magnesium ions continued to be accumulated.

A variety of theories have been proposed relating to the metabolic control of glycolysis. Certainly, the regeneration of inorganic phosphorus and ADP via biosynthesis and ATPase activities are important constraints in both the Embden-Meyerhof and Entner-Doudoroff pathways as is the regeneration of NAD^+. Embden-Meyerhof glycolysis appears to be under considerable allosteric control being responsive to energy charge, activators, and inhibitors (Akerman, 1985; Bosca and Corredor, 1984; Cooper, 1978; Fraenkel, 1981 and 1982). However, the Entner-Doudoroff pathway in Z. mobilis lacks two key allosteric enzymes, phosphofructokinase and an allosteric hexokinase. Metabolite levels in Z. mobilis appear very low and Algar and Scopes (1985) have proposed that little allosteric regulation operates within this pathway during glycolysis. With regard to the enzymes of Embden-Meyerhof glycolysis, it is generally thought that most if not all exert partial control over glycolytic flux with no single rate-limiting step (Crabtree and Newsholme, 1985; Fell and Sauro, 1985; Kascar and Burns, 1973). However, the excellent correlations of glycolytic flux (fermentative activity) with the levels of pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase at various stages of fermentation provide evidence that this may not be true for the Entner-Doudoroff pathway in Z. mobilis.

It is interesting to note that pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase are present in both major glycolytic pathways and both are typically represented in Embden-Meyerhof organisms such as S. cerevisiae as isozymes (McAlister and Holland, 1985), implying a degree of importance and regulation. Although no detailed studies have been published with this enzyme from *Z. mobilis*, in other organisms pyruvate kinase is subject to "feed forward" activation by fructose-1,6-diphosphate, phosphoenolpyruvate, and other glycolytic intermediates and is inhibited by one of its products, ATP (Cooper, 1978; Kotlarz et al., 1975; Thompson and Torchia, 1984). Pyruvate kinase has been proposed as a principal determinant of glycolytic flux in *Streptococcus* (Thompson and Torchia, 1984), *Viellonella parvula* (Ng and Hamilton, 1975), *Propionibacterium shermanii* (Smart and Pritchard, 1982), and in *S. cerevisiae* (Maitra and Lobo, 1977). This enzyme catalyzes the thermodynamically favorable production of ATP and pyruvate from phosphoenolpyruvate, serving to pull unfavorable reactions forward in glycolysis. A reduction in the level of this enzyme has been shown to cause an increase in the levels of phosphoenolpyruvate and phosphoglycerate in *S. lactis* (Thompson and Torchia, 1984) and in *S. cerevisiae* (Fraenkel, 1981). Kinetic studies with *S. lactis* provide strong evidence for the importance of this allosteric regulation in determining glycolytic flux and the partitioning of phosphoenolpyruvate between biosynthetic functions and energy generation (Thompson, 1978; Thompson and Torchia, 1984).

Glyceraldehyde-3-phosphate dehydrogenase is one of the most abundant enzymes in *S. cerevisiae* and can constitute up to 10% of the soluble proteins during growth with glucose

(Fraenkel, 1981). This activity is encoded by at least three structural genes in S. cerevisiae (McAlister and Holland, 1985). Little is known concerning this enzyme in Z. mobilis although activities in extracts are at least 4-fold that required to support glycolysis. This enzyme catalyzes a thermodynamically unfavorable reaction which is coupled to subsequent steps is glycolysis.

Although many of the enzymes for hexose metabolism are known to be inducible in metabolically versatile organisms such as yeast (Fraenkel, 1981) and Pseudomonas (Lessie and Phibbs, 1984), obligately fermentative organisms with limited substrate ranges like Z. mobilis would not be expected to exhibit a similar degree of control. In fermenting yeast (Fraenkel, 1981) and in Z. mobilis (Algar and Scopes, 1985), 30% to 50% of the cellular protein is devoted to glycolytic enzyme activities, emphasizing the high rate of metabolism required for competitive energy production by such inefficient processes. It is interesting to note that all the activities of the glycolytic enzymes in Z. mobilis were increased at a rate in excess of that of bulk protein (increasing specific activity). This is consistent with the high activity of promoters associated with many of the cloned glycolytic enzymes in other systems (Branlant et al., 1983). In Z. mobilis, two patterns of enzyme production were noted for the glycolytic enzymes. The activity of pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase began to fall at the end of exponential growth although the production of the remaining glycolytic enzymes continued during the transition phase until the end of growth. Many of the genes in hexose metabolism have been shown to be clustered in *E. coli* and in *Pseudomonas* and in some cases under a common transcriptional control (Cuskey et al., 1985; Fraenkel, 1981). The clustering of the glycolytic enzymes such as pyruvate kinase and glyceraldehyde-3-phosphate dehydrogenase within an operon in *Z. mobilis* would provide an efficient means to regulate their coordinate expression.

CHAPTER IV
CONCLUSIONS

Zymomonas mobilis produces ethanol from glucose at a faster rate than *S. cerevisiae*, with 98% efficiency (Rogers et al., 1982; Montenecourt, 1985). However, like yeast, the accumulation of ethanol during batch fermentation results in a progressive decline in the fermentative activity of the cells (Aiba et al., 1968; Nagodawithana and Steinkraus, 1976; Laudrin and Goma, 1982; Moulin et al., 1984; Ingram and Buttke, 1984). To understand the physiological and the biochemical bases by which ethanol inhibits the fermentation process, this study has divided the alcohol effects on *Z. mobilis* into two parts: immediate effects of the cells upon exogenous addition of ethanol (acute effects); and the more complex intracellular physiological changes occurring during batch fermentation.

The acute effects of ethanol on the rate of fermentation have been examined by the addition of alcohol to active cells of *Z. mobilis* taken at late exponential phase of growth. The rate of fermentation was measured as the rate of consumption of glucose in growth medium and phosphate buffer (pH 6.0) at 30°C. The following conclusions have been drawn from these studies:

1. Ethanol caused a dose-dependent inhibition of fermentation in vivo, i.e., as the added ethanol concentration was increased from zero to 4.4 M, the amount of inhibition of fermentation was increased from zero to 100%, in a linear relationship.

2. Removal of ethanol by centrifugation (washing) did not immediately reverse the inhibitory effects on the rate of fermentation, although Z. mobilis is freely permeable to ethanol. However, incubation of the washed cells in growth medium for one and a half hour improved their fermentative activity by about 60% in the absence of measurable growth. The rate of fermentation of the cells incubated in buffer only continued to decrease. These experiments demonstrated that growth medium helped the cells to recover their activity and protected the cells against ethanol damage more than phosphate buffer, which rendered the cells more sensitive to ethanol.

3. Among the components of the growth medium, only yeast extract was shown to be beneficial for the reversal of ethanol damage to the cells. Further partitioning the yeast extract demonstrated that only the inorganic (ashed) part restored fermentative activity. Magnesium ion was discovered to be the protective agent in yeast extract.

4. Ethanol caused the leakage of small cofactors (magnesium ions) and coenzymes (nucleotides) through the plasma membrane. The loss of magnesium and coenzymes deprived the glycolytic and alcohologenic enzymes of an

essential components to carry out their functions in the cellular metabolism. The loss of these molecules may have led the cells to slow down their rate of fermentation.

5. The addition of magnesium ions alone to ethanol-damaged cells restored their fermentative activity. A similar addition of magnesium ions plus a mixture of nucleotides to cells exposed to 4.4 M ethanol improved their fermentative activity by 30%. Therefore, 4.4 M ethanol did not completely damage the fermentative activity of the glycolytic enzymes.

6. Ethanol damage to fermentation in _Z. mobilis_ results from damage to the cell membrane decreasing its effectiveness as a permeability barrier and increasing the leakage of essential coenzymes and cofactors.

The second part of this study was designed to examine more thoroughly the complex physiological changes occurring in the cells during the course of batch fermentation using 20% glucose. The fermentative activity of the cells was measured as umoles CO_2 evolved per mg cell protein per hour. It was obvious that the fermentation rate peaked after 18 h and declined rapidly thereafter. Even after the removal of accumulated ethanol (1.1% w/v) and resuspension in fresh medium the shape of the curve stayed the same. This early decline in fermentative activity reflected a permanent physiological changes in the cell rather than, the presence of ethanol, or the lack of sugar substrate.

To gain a better understanding of this early decline in the rate of fermentation, the levels of many of the intracellular components required for fermentation were measured. The data clearly indicated that:

1. The intracellular concentration of magnesium peaked at 30 h; the ATP level remained relatively constant for the first 30 h, the ADP level declined progressively after 12 h, and the AMP level was low initially but increased rapidly after 36 h. Also the energy charge of the cells remained high for the first 30 h. Thus none of these factors are major determinants in the early decline of the rate of fermentation.

2. The level of NAD^+, the NAD^+/NADH ratio, and total nicotinamide adenine nucleotides peaked at 18 h and declined thereafter, coincident with the observed changes in fermentative activity.

3. The internal pH was highest at 18 h (pH 6.3) and declined to pH 5.3 after 30 h. In the same time the ΔpH peaked at 18 h (1.5) and dropped to 0.5 units after 30 h, coincident with the observed changes in the rate of fermentation.

4. Ten of the twelve enzymes in Entner-Doudoroff pathway peaked at 30 h, well beyond the peak of fermentative activity (18 h).

5. The fermentative activity of _Z_. _mobilis_ cells exhibited an excellent correlation with the specific

activity of two glycolytic enzymes, pyruvate kinase, and glyceraldehyde-3-phosphate dehydrogenase.

6. ^{31}P-NMR studies revealed the accumulation of glyceraldehyde-3-phosphate, 3-phosphoglyceric acid, KDPG, and 6-phosphogluconate in cells taken at 30 h and very low levels of these monophosphate sugars in more active cells taken at 18 h. This accumulation of sugar phosphates is consistent with the hypothesis that pyruvate kinase and/or glyceraldehyde-3-phosphate dehydrogenase are restricting the glycolytic flux.

7. The viable cell number (colony forming units) peaked at 30 h and declined thereafter. Exponential growth, however, ceased after 18 h. The onset of the end of exponential growth coincided with the beginning of decline in fermentation rate.

In summary, the decline in the rate of fermentation can be divided into two parts. An early decline after 18 h (1.1% w/v accumulated ethanol), and a late decline after 30 h (6.2% w/v accumulated ethanol). The early decline was associated with the decline in the internal pH, ΔpH, NAD^+, NAD^+/NADH ratio, pyruvate kinase, and glyceraldehyde-3-phosphate dehydrogenase. The late decline was associated with cell death, leakage of magnesium, degradation of nucleotides, and possibly nucleic acid, and the turnover of the glycolytic enzymes.

It is clear that the decline in fermentative activity in Z. mobilis during batch fermentation does not result solely from the inhibitory action of ethanol. But it is possible that a combination of the above mentioned factors contribute to inhibition at different stages of cellular growth and fermentation. Further studies should provide conclusive evidence to identify which of these physiological changes were most detrimental to the fermentative activity during batch fermentation.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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